A Conserved Non-Reproductive GnRH System in Chordates

Takehiro G. Kusakabe1,2*, Tsubasa Sakai3, Masato Aoyama3, Yuka Kitajima2, Yuki Miyamoto1,2, Toru Takigawa1, Yutaka Daido1, Kentaro Fujiwara1, Yasuko Terashima1,2, Yoko Suguchi1, Giorgio Matassii1,4, Hitoshi Yagisawa2, Min Kyun Park5, Honoo Satake3, Motouyuki Tsuda2,6*

1Department of Biology, Faculty of Science and Engineering, Konan University, Kobe, Japan, 2Graduate School of Life Science, University of Hyogo, Kamigori, Hyogo, Japan, 3Division of Biomolecular Research, Suntory Institute for Bioorganic Research, Shimamoto, Osaka, Japan, 4Department of Agriculture and Environmental Sciences, University of Udine, Udine, Italy, 5Department of Biological Sciences, Graduate School of Science, the University of Tokyo, Tokyo, Japan, 6Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University, Sanuki, Kagawa, Japan

Abstract

Gonadotropin-releasing hormone (GnRH) is a neuroendocrine peptide that plays a central role in the vertebrate hypothalamo-pituitary axis. The roles of GnRH in the control of vertebrate reproductive functions have been established, while its non-reproductive function has been suggested but less well understood. Here we show that the tunicate Ciona intestinalis has in its non-reproductive larval stage a prominent GnRH system spanning the entire length of the nervous system. Tunicate GnRH receptors are phylogenetically closest to vertebrate GnRH receptors, yet functional analysis of the receptors revealed that these simple chordates have evolved a unique GnRH system with multiple ligands and receptor heterodimerization enabling complex regulation. One of the gnrh genes is conspicuously expressed in the motor ganglion and nerve cord, which are homologous structures to the hindbrain and spinal cord of vertebrates. Correspondingly, GnRH receptor genes were found to be expressed in the tail muscle and notochord of embryos, both of which are phyleotypic axial structures along the nerve cord. Our findings suggest a novel non-reproductive role of GnRH in tunicates. Furthermore, we present evidence that GnRH-producing cells are present in the hindbrain and spinal cord of the medaka, Oryzias latipes, thereby suggesting the deep evolutionary origin of a non-reproductive GnRH system in chordates.

Introduction

Gonadotropin-releasing hormone (GnRH) plays a pivotal role in controlling reproductive functions in vertebrates. Three major GnRH systems have been characterized in vertebrates: the conventional hypophysiotropic GnRH (GnRH1) system and two extrahypothalamic GnRH (GnRH2 and GnRH3) systems [1–4]. The hypothalamic GnRH1 regulates the reproductive endocrine system by facilitating the release of gonadotropins from the pituitary. The cell bodies of the GnRH2 and GnRH3 systems are localized in the midbrain and the terminal nerves, respectively. Whether hypophysiotropic or extrahypothalamic, most functions of the GnRH systems have been implicated in the control of reproductive activities, such as gonad development, gonad function, and reproductive behaviors [2,3]. Non-reproductive roles of GnRH have been suggested by the broad distribution of GnRH receptors (GnRHRs) in the central nervous system [5–8] as well as by projection of GnRH neurons to wide areas in the brain [3,9]. Furthermore, the presence of GnRH in early embryos and experimental manipulation of GnRH activity suggest developmental roles of GnRH in vertebrate embryos [10–14]. Compared to its reproductive roles, however, the non-reproductive roles of GnRH are less well understood.

Tunicates are the sister group of vertebrates [15,16]. It has been suggested that GnRH is involved in reproductive control in sessile tunicates, namely ascidians [17–19]. Despite their simplicity in anatomical organization and reproductive mode, GnRHs and their receptors exhibit a rich diversity in tunicates; six GnRH peptides and four receptors are encoded by the genome of the ascidian Ciona intestinalis [19–21]. This molecular complexity of the GnRH system leads us to hypothesize a non-reproductive role of GnRH in ascidians as well. Such a non-reproductive role of GnRH has been suggested by the presence of transcripts encoding GnRH in Ciona embryos and larvae [19]. GnRH-like immunoreactivity in adult sensory neurons of some species also suggests non-reproductive roles of GnRH in ascidians [22,23].

The roles and localization of GnRHs have been investigated in adult tunicates, which exhibit an extremely modified form as chordates, while little is known about GnRH systems in the larvae, which exhibit a well-conserved chordate body plan. Here we show a novel non-reproductive feature of the GnRH system in C. intestinalis larvae. Our results suggest that tunicates evolved a sophisticated GnRH system with specific interactions between...
multiple ligands and receptors. The GnRH genes are conspicuously expressed in the central nervous system (CNS) through the entire antero-posterior body axis. Correspondingly, the GnRH receptor genes are specifically expressed in the phylotypic axial structures along the CNS. We further present evidence for localization of cells producing GnRH in the hindbrain and spinal cord of vertebrates. Our findings demonstrate that the GnRH system is deeply associated with the chordate-specific characteristics and suggest the evolutionary conservation of a non-reproductive GnRH system between tunicates and vertebrates.

Results

Prominent Expression of gnrh Genes in the Ciona intestinalis Larva

Six GnRH peptides, tGnRH-3 to -8, are encoded by two genes, Ci-gnrh1 and Ci-gnrh2 in C. intestinalis (Fig. S1) [19]. In previous studies, localization of GnRHs or their mRNAs in ascidians were examined in adult tissues [17,19,24–26]. Expression of the Ci-gnrh1 and Ci-gnrh2 genes has been demonstrated during larval development by RT-PCR [19], but their spatial expression patterns have not been reported. To elucidate potential sites utilizing GnRH signaling in ascidian larvae, we examined the spatial expression patterns of Ci-gnrh1 and Ci-gnrh2 in C. intestinalis larvae by whole-mount in situ hybridization. Both genes were found to be specifically but distinctly expressed in the nervous system of the larva (Fig. 1A, B). Ci-gnrh1 is strongly expressed in a population of cells in the brain vesicle, the anterior portion of the central nervous system (Fig. 1A, C). Transcripts of Ci-gnrh1 were also detected in the motor ganglion (also called “visceral ganglion”; we adopt here a terminology recently proposed by Nishino et al. [27]), but its expression level was much lower than that in the brain vesicle (Fig. 1C). In addition to the expression in the central nervous system, Ci-gnrh1 is also expressed in parts of the peripheral nervous system; it is expressed in a few cells in the adhesive organ and a subpopulation of rostral epidermal neurons (RTENs) (Fig. 1C). The cells expressing Ci-gnrh1 in the adhesive organ are likely to be papillar neurons, as they possess long neurites (probably axons) as visualized by GFP under the control of the cis-regulatory region of Ci-gnrh1 (Fig. 1D). The Ci-gnrh1:gfp fusion construct could also visualize the brain vesicle and the motor ganglion (Fig. 1D).

The expression of Ci-gnrh2 was detected mostly in the central nervous system. The most conspicuous expression site of Ci-gnrh2 was the caudal nerve cord; it was expressed throughout the entire nerve cord from the posterior trunk to the tail tip (Fig. 1B). The brain vesicle and the motor ganglion also contained cells expressing Ci-gnrh2 (Fig. 1B, E). In the brain vesicle, there are at least two populations of Ci-gnrh2-expressing cells: one is located close to the ocellus pigment cell, while the other is located in the posterior brain region (Fig. 1E). Expression of GFP under the control of the cis-regulatory region of Ci-gnrh2 recapitulated the endogenous gene expression pattern (Fig. 1G). In some specimens, Ci-gnrh2:gfp visualized a neuron in the motor ganglion, which had a neurite (presumably an axon) extending into the tail along the nerve cord (Fig. 1H).

Four GnRH Receptor Paralogs in C. intestinalis

Four GnRH receptors (Ci-GnRHR1, Ci-GnRHR2, Ci-GnRHR3, and Ci-GnRHR4) are encoded in the genome of C. intestinalis [20,21] (Fig. 2A). We previously identified and characterized Ci-GnRHR1 and Ci-GnRHR2 [20], and Tello et al. [21] reported identification of Ci-GnRHR3 and Ci-GnRHR4 along with functional characterization of the four C. intestinalis GnRH receptors. In this study, we independently isolated full-length cDNA clones for Ci-GnRHR3 and Ci-GnRHR4. The Ci-GnRHR4 cDNA clone we isolated contained an additional exon at the 3′ end, resulting in an extended coding sequence for a 411 amino acids polypeptide as compared to the previously reported 366 amino acids polypeptide [21]. The positions of introns, including this newly identified intron, are conserved among all C. intestinalis GnRH receptor genes (Fig. S2). Another new finding is that the 5′ ends of the Ci-GnRHR3 and Ci-GnRHR4 cDNAs have a spliced leader sequence (5′-ATTCTATTTTGAATAAG-3′), which is identical to that found in the C. intestinalis troponin I gene [28]. The nucleotide sequences of the Ci-GnRHR3 and Ci-GnRHR4 cDNAs have been deposited in the DDBJ, EMBL, and GenBank Nucleotide Databases under the accession numbers AB540990 and AB540991, respectively.

There are a number of amino acid differences between the previously reported sequences of Ci-GnRHR3 and Ci-GnRHR4 obtained from a north Atlantic population in Woods Hole, MA [21] and the present sequences obtained from a Pacific population in Japan; eighteen and 7 amino acids were different in Ci-GnRHR3 and Ci-GnRHR4, respectively (Fig. S2). Similar extent of sequence divergence between North Atlantic and Pacific populations was also reported for Ci-GnRHR1 and Ci-GnRHR2 [21] (Fig. S2). Recent studies have revealed the existence of two cryptic species: C. intestinalis sp. A, distributed in the Mediterranean, northeast Atlantic, and Pacific, and C. intestinalis sp. B, found in the North Atlantic [29]. Thus, the observed divergence between the two sets of Ci-GnRHR sequences is probably attributable to the genetic variations between C. intestinalis sp. A and C. intestinalis sp. B.

Molecular Phylogeny of the GnRHR Family

Roch and colleagues recently reported a large-scale phylogenetic analysis of GnRHRs [30]. We also examined the phylogenetic relationships among GnRHRs, and compared our results with the previous study [30]. The protein dataset analyzed consisted of 55 receptor sequences, including two new lamprey GnRHRs, and the human oxytin and vasopressin V2 receptors, used as outgroups (Table S1). Multiple sequence alignment was performed using TM-Coffee [31], which is specifically designed to align transmembrane proteins. Only the aligned residues with a high confidence index were retained (see Materials and Methods). Maximum Likelihood (ML) phylogenetic inference was carried out using PhyML v3 [32], Lefaphy v1.0 [33] and RAxML v7.2.8 [34], for these programs use different tree-space search algorithms. The three strategies converged on the same topology, which is shown in Fig. S3. See Materials and Methods for details.

Our results are largely consistent with the previous report [30]. First, GnRH receptors form a superfamily with corazonin (Crz) and adipokinetic hormone (AKH) receptor families. Second, the four GnRH receptors in C. intestinalis belong to a paralogous cluster (possibly tunicate-specific). Third, there are two distinct lineages of amphioxus GnRH receptors [35], one (amphioxus GnHR1) and 2) closely related to the vertebrate and tunicate GnRH receptors and the other (amphioxus GnHR3 and 4) grouped together with protostome GnHR and corazonin receptors. Fourth, diversification of GnRH receptors occurred independently in each chordate subphylum, although we could not statistically resolve interrelationships among vertebrate, tunicate, and cephalochordate GnHRs.

A major difference between the present result and the one by Roch et al. [30] is the position of sea urchin GnRHRs. These authors proposed that sea urchin GnRHRs are orthologs of CrzRs/GnRHRs. In contrast, in our analysis, the three sea urchin
receptors lie basal to the chordate clade. We consider this topology more likely than the alternative topology (i.e., GnRH + AKHR/ACPR clade, Fig. 5 in [30]) given the high log-likelihood difference (∆logL = 9.43) between our topology and the one proposed by Roch et al., though the latter cannot be excluded on statistical grounds (see Materials and Methods).

Figure 1. Expression of gnrh genes in the Ciona intestinalis larvae. Spatial expression patterns of Ci-gnrh1 (A, C, and D) and Ci-gnrh2 (B, E, G, and H) in a whole body (A, B, and G) or trunk region (C-F, and H) of C. intestinalis larvae. The gene expression was visualized by whole-mount in situ hybridization (A, B, C, and E) or by GFP reporter expression under the control of the regulatory region of Ci-gnrh1 (D, G, and H). (F) An unstained control larva showing the otolith (ot) and ocellus (oc) pigment cells in the brain vesicle. White arrowheads in (D) indicate GFP-positive axons of papillar neurons (pn). The white arrowhead in (H) indicates a GFP positive neurite. bv, brain vesicle; nc, nerve cord; rten, rostral trunk epidermal neuron; mg, motor ganglion. Scale bars: 100 μm in (A); 50 μm in (C). doi:10.1371/journal.pone.0041955.g001
Non-Reproductive GnRH System in Chordates

A

Gene Model
ci0100133065
ci0100153146
ci0100152622

[Scaffold 93] Ci-GnRHR3 (15.9 kb) Ci-GnRHR2 (7.6 kb) Ci-GnRHR1

Gene Model

[Scaffold 198] Ci-GnRHR4

ci0100134571

B

Ci-GnRHR1

C

Ci-GnRHR2

D

Ci-GnRHR3

E

Ci-GnRHR4

F1

Ci-GnRHR2

F2

Ci-GnRHR2

F3

Ci-GnRHR2

G

mu

Ci-GnRHR1::gfp

H

mu

Ci-GnRHR2::gfp

I

no

Ci-GnRHR3::gfp
**Figure 2.** The *C. intestinalis* GnRH receptors (GnRHRs) and spatial expression patterns of *gnhr* genes in the larva. (A) Genomic organization of the *C. intestinalis* GnRHRs. Three GnRHRs (Ci-GnRHR1, Ci-GnRHR2, and Ci-GnRHR3) are encoded by a gene cluster located on Scaffold 93 while the gene encoding another GnRHR (Ci-GnRHR4) is located in Scaffold 198. Arrows indicate the location and orientation of the genes. Gene model IDs in the JGI version 1.0 genome database are indicated below the arrows. (B) Whole larvae showing localization of transcripts of Ci-GnRHR1 (B), Ci-GnRHR2 (C), Ci-GnRHR3 (D), or Ci-GnRHR4 (E). (F1–F3) A middle part of the tail of the same individual with different focal planes showing muscle cells and caudal epidermal neurons (cen) expressing Ci-GnRHR2. (G–I) GFP reporter expression in the tail muscle (mu) or notochord (no) under the control of the regulatory region of Ci-GnRHR1 (G), Ci-GnRHR2 (H), or Ci-GnRHR3 (I). cen, caudal epidermal neuron; mu, muscle; no, notochord. Scale bars: 100 μm in (B), 50 μm in (F1) and (G).

doi:10.1371/journal.pone.0041955.g002

**Ciona GnRHRs** are expressed in Phylotypic Axial Structures

The conspicuous expression of *Ci-gnrh*1 and *Ci-gnrh*2 in the larval stage suggests that their receptors are also present and function at the larval stage in previous studies, however, spatial expression patterns of genes encoding Ci-GnRHRs was only examined in adult tissues [20,21]. We, therefore, examined spatial expression patterns in the larva of *Ci-GnRHR1, Ci-GnRHR2, Ci-GnRHR3*, and *Ci-GnRHR4* by whole-mount in situ hybridization. All *Ci-GnRHR* genes are expressed in the brain vesicle (Fig. 2B, C, D, E), *Ci-GnRHR1, Ci-GnRHR2*, and *Ci-GnRHR3* are also expressed in the motor ganglion (Fig. 2B, C, D). In addition, *Ci-GnRHR1* and *Ci-GnRHR2* are expressed in the adhesive organ and the tail muscle cells (Fig. 2B, C, F). *Ci-GnRHR2* is also expressed in some cells located on the dorsal and ventral midlines of the tail (Fig. 2C, F). These midline cells seem to be caudal epidermal neurons, which express a glutamatergic marker *Ci-VGLUT* [36]. *Ci-GnRHR3* is also expressed in the tail, but the expression pattern is different from those of *Ci-GnRHR1* and *Ci-GnRHR2*; its expression is more evident in the proximal (anterior) than distal region of the tail (Fig. 2D). The cells expressing *Ci-GnRHR3* in the tail are distributed along the cavity of the notochord in a similar pattern to that of nuclei in the mature notochord [37]. The expression of *Ci-GnRHR1* and *Ci-GnRHR2* in muscle cells and *Ci-GnRHR3* in notochord cells was further supported by GFP reporter expression under the control of the cis-regulatory region of each gene (Fig. 2G, H, I). In summary, *Ciona GnRHRs* are conspicuously expressed in phylotypic axial structures, including the central nervous system, notochord, and paraxial muscle.

**Signaling and Regulation of Ciona GnRHRs**

We confirmed the previous report [21] that in COS-7 cells Ci-GnRHR1 activates IP generation in response to tGnRH-6 (Table S2). We also found that tGnRH-7 and tGnRH-8 were able to stimulate IP accumulation in COS-7 cells expressing Ci-GnRHR1, but yet with relatively high EC50 values (Table S2). In HEK293-MSR cells expressing Ci-GnRHR1, in which elevation of intracellular Ca2+ is induced with much lower concentrations of tGnRHs than IP generation in COS-7 cells [38], tGnRH-6 evoked the most potent elevation of intracellular Ca2+, whereas other tGnRHs showed 10-fold to 1000-fold lower activities (Table 1; Fig. S4A). No significant Ca2+ increase was observed in cells expressing Ci-GnRHR2, 3, or 4 in response to any GnRHS. These results confirmed that Ci-GnRHR1 exclusively stimulates mobilization of intracellular Ca2+ with high specificity to tGnRH-6.

Activation of the cAMP pathway by tGnRHs has been reported as another cellular response in COS-7 cells expressing Ci-GnRHRs [21]. We examined induction of cAMP production by each combination of tGnRH and Ci-GnRHR in HEK293-MSR cells. Our results obtained using HEK293-MSR cells (Table 2; Fig. S1B, C, D) are compatible with the previous report using COS-7 cells [21], indicating the ligand-selective cAMP production mediated by Ci-GnRHR1, Ci-GnRHR2, and Ci-GnRHR3. We also confirmed that Ci-GnRHR4 was devoid of cAMP production or inhibition upon application of any tGnRHs (data not shown).

We recently demonstrated that Ci-GnRHR4 could form a heterodimer with Ci-GnRHR1 and Ci-GnRHR2 in vivo and modulate signaling via these receptors [38,39]. Here we further evaluated the specificity of the modulator function of Ci-GnRHR4. In the cells co-transfected with Ci-GnRHR1 and Ci-GnRHR4, tGnRH-6 exhibited approximately 10-fold more potent activity on intracellular calcium mobilization than in the cells expressing Ci-GnRHR1 alone, whereas the activities of other tGnRHs were not up-regulated (Fig. S4E and Table 2). In contrast, co-expression of Ci-GnRHR1 and Ci-GnRHR4 resulted in no significant alteration of pharmacological profiles of cAMP production, although the maximal levels of cAMP by tGnRH-6 and tGnRH-8 were slightly increased (Fig. S4F).

**Table 1.** EC50 values (nM) of tGnRHs for intracellular calcium ion mobilization in HEK293-MSR cells expressing only Ci-GnRHR1 or co-expressing Ci-GnRHR1 and 4.

|          | Ci-GnRHR1 | Ci-GnRHR1&4 |
|----------|-----------|-------------|
| tGnRH-3  | >100      | >100        |
| tGnRH-4  | >100      | >100        |
| tGnRH-5  | >100      | >100        |
| tGnRH-6  | 6.971     | 0.55        |
| tGnRH-7  | 100       | 88.8        |
| tGnRH-8  | >100      | >100        |

The EC50 values of tGnRH-6 are underlined to emphasize the dramatic increase in the potency on the cells co-transfected with Ci-GnRHR1 and 4.

doi:10.1371/journal.pone.0041955.t002

**Table 2.** EC50 values (nM) of t-GnRHs for cAMP production in HEK293-MSR cells expressing only Ci-GnRHR1, 2, 3 or co-expressing Ci-GnRHR1 and 4, or Ci-GnRHR3 and 4.

|          | Ci-GnRHR1 | Ci-GnRHR2 | Ci-GnRHR3 | Ci-GnRHR1&4 | Ci-GnRHR3&4 |
|----------|-----------|-----------|-----------|-------------|-------------|
| tGnRH-3  | >100      | >100      | 19.5      | >100        | 19.1        |
| tGnRH-4  | >100      | >100      | >100      | >100        | >100        |
| tGnRH-5  | >100      | >100      | 9.19      | >100        | 6.09        |
| tGnRH-6  | 0.334     | >100      | >100      | 0.318       | >100        |
| tGnRH-7  | 34        | 29.2      | >100      | 27.6        | >100        |
| tGnRH-8  | 31        | 8.78      | >100      | 19.9        | >100        |

No markedly altered effects of co-expression with Ci-GnRHR4 on activities of tGnRHs were detected.

doi:10.1371/journal.pone.0041955.t003

GnRHRs. Three GnRHRs (Ci-GnRHR1, Ci-GnRHR2, and Ci-GnRHR3) are encoded by a gene cluster located on Scaffold 93 while the gene encoding another GnRHR (Ci-GnRHR4) is located in Scaffold 198. Arrows indicate the location and orientation of the genes. Gene model IDs in the JGI version 1.0 genome database are indicated below the arrows. (B-E) Whole larvae showing localization of transcripts of Ci-GnRHR1 (B), Ci-GnRHR2 (C), Ci-GnRHR3 (D), or Ci-GnRHR4 (E). (F1–F3) A middle part of the tail of the same individual with different focal planes showing muscle cells and caudal epidermal neurons (cen) expressing Ci-GnRHR2. (G–I) GFP reporter expression in the tail muscle (mu) or notochord (no) under the control of the regulatory region of Ci-GnRHR1 (G), Ci-GnRHR2 (H), or Ci-GnRHR3 (I). cen, caudal epidermal neuron; mu, muscle; no, notochord. Scale bars: 100 μm in (B), 50 μm in (F1) and (G).
cells expressing Ci-GnRHR3 alone (Fig. S4G and Table 2). Thus, the regulatory function of Ci-GnRHR1 is specific to a ligand, partner receptor, and intracellular signaling pathway.

**Putative GnRH-producing Neurons in the Vertebrate Hindbrain and Spinal Cord**

Unexpectedly conspicuous expression of Ci-gnrh2 in the motor ganglion and the nerve cord motivated us to ask whether similar expression of gnrh genes are found in vertebrates. To the best of our knowledge, however, the GnRH-producing cell bodies have not been reported in the vertebrate hindbrain and spinal cord. We adopted medaka *O. latipes* as a vertebrate model because both its GnRHs and their receptors have been well characterized [40,41] and cells expressing genes of interest are easily visualized in living animals by promoter transgenics [42,43]. Among the three genes (gnrh1, gnrh2, and gnrh3) encoding GnRH in medaka, we focused on gnrh2 for the following two reasons. First, embryonic expression has been reported for gnrh1 and gnrh3 [44], but not for gnrh2. Second, chicken GnRH-II, which is encoded by the medaka gnrh2 gene [40], can effectively activate Ci-GnRHRs [21]. We isolated a 5-kb upstream region of medaka gnrh2 and used it to drive the GFP reporter in medaka embryos. Three days after fertilization, GFP fluorescence specifically appeared in the central nervous system, including the hindbrain and spinal cord (Fig. 3A). In the hindbrain, cell bodies of some reticulospinal neurons were labeled with GFP (Fig. 3B). Their axons that extend into spinal cord were also labeled (Fig. 3B). In the spinal cord, at least two types of neurons were labeled. One type includes neurons with a primary axon that first extends ventrally, turns rostrally in the ventral spinal cord, and then ascends on the ipsilateral side of the spinal cord among other axons in the marginal zone (Fig. 3C). The morphology of this type of neurons closely resembles that of V1 inhibitory interneurons [45–47]. Labeled neurons of the other type were commissural neurons whose axons extended across the ventral midline towards contralateral sides (Fig. 3D). Neurons of the former type were more abundant among GFP-positive neurons; they distributed throughout the entire spinal cord along the antero-posterior axis. The expression of gnrh2 in the hindbrain and spinal cord of the medaka was further confirmed by RT-PCR. Transcripts of gnrh2 were detected in the midbrain, hindbrain and spinal cord, while those of gnrh1 and gnrh3 were only detected in the forebrain and midbrain (Fig. 3E). The results suggest that cells expressing a gnrh gene are present in the vertebrate hindbrain and spinal cord.

**Discussion**

**Complexity of the GnRH System in Tunicates**

Two or more GnRH peptides and their receptors have been reported in most animal species studied [48–50]. Among these, the tunicate *C. intestinalis* is particularly striking for the multiplicity of GnRH ligands and receptors. In the present study, we showed that the genes encoding all these peptides and receptors were conspicuously expressed in the larvae. Expression patterns of these genes are distinct from each other. The differential expression of paralogous genes adds further complexity to the tunicate GnRH system.

Tello et al. [21] reported selective activation of Ci-GnRHRs by different t-GnRHs, and previous works, we demonstrated that Ci-GnRHR4 could modulate Ci-GnRHR1 and Ci-GnRHR2 signaling via heterodimerization [38,39]. The present study confirmed these previous findings and further extended the understanding of the selectivity and specificity of these molecular interactions. Expression patterns of Ci-GnRHR and Ci-gnrh genes are consistent with receptor-ligand selectivity. For example, Ci-GnRHR1 and Ci-GnRHR2 in muscle cells can be activated by tGnRH-7 and tGnRH-8, which are encoded by Ci-gnrh2 expressed in the nerve cord. Ci-gnrh1 encodes tGnRH-6, which is the most potent ligand for Ci-GnRHR1 and a moderate activator for Ci-GnRHR2; consistently, Ci-gnrh1, Ci-GnRHR1, and Ci-GnRHR2 are all expressed in the adhesive organ and in the brain vesicle. Ci-gnrh1 also encodes tGnRH-3 and tGnRH-8, potential ligands for Ci-GnRHR3. Ci-GnRH is expressed in the brain vesicle, motor ganglion, and anterior notochord, which are reasonably close to the expression sites of Ci-gnrh1. The consistency between expression patterns and effective ligand-receptor combinations suggests the functional significance of GnRH signaling in the ascidian larva.

Ci-GnRHR4 is a positive regulator for Ci-GnRHR1 in the generation of intracellular calcium ions by interaction with tGnRH-6. The action of Ci-GnRHR4 is specific with respect to heterodimer partners, ligands, and intracellular signaling pathways. Superimposition of possible sites of Ci-GnRHR1 modulation by Ci-GnRHR1 onto the expression patterns of the two receptors can increase the regulatory complexity of GnRH signaling in the ascidian larva. For example, Ci-GnRHR1 and Ci-GnRHR4 are co-expressed in the brain vesicle, whereas only Ci-GnRHR1 is expressed in the adhesive organ, motor ganglion, and muscle cells. Thus, ligand-receptor selectivity, differential gene expression, and receptor heterodimerization generate a vast diversity of GnRH signaling modes at different sites of the larva.

**Non-reproductive Features of the GnRH System in *Ciona***

The larvae of *C. intestinalis* occupy a non-reproductive dispersal stage; the gonad develops after metamorphosis and reproduction occurs much later [51,52]. During metamorphosis, the nervous system undergoes extensive reorganization [53] and the tail, containing the nerve cord, muscle, notochord, and caudal epidermis, degenerates [54,55]. Conspicuous expression of the genes for GnRHs and GnRHRs in the nervous system and tail tissues in the larva strongly suggests that GnRHs regulate biological processes that are not directly related to reproduction. What kind of biological processes do they regulate? Regulation of locomotion and swimming behavior is one possibility. Two types of environmental signal, light and gravity, regulate the swimming behavior of the larva [56–59]. Light and gravity are sensed by the ocellus and otolith, respectively, in the brain vesicle, and the sensory inputs are then processed in the posterior brain vesicle and motor ganglion to regulate muscle contraction in the tail [27,60]. Since the receptor genes are expressed in the brain vesicle, motor ganglion, and muscle cells, and the ligand genes are expressed in the brain vesicle, motor ganglion and nerve cord, GnRH signaling would be able to regulate sensory reception, neuronal processing, and muscle functions.

Another possible role of GnRH in the ascidian larva may be the control of metamorphosis. The initiation of metamorphosis is triggered by settlement on substrates with papillae of the adhesive organ [54,55,61]. Stimuli of settlement sensed by the adhesive organ are thought to be transmitted to the central nervous system to proceed with metamorphic events in the trunk and tail tissues. When adhesive papillae of *Ciona* larvae have been removed, tail absorption, a major metamorphic event, is suppressed and the tail retains its swimming ability [55]. Ci-gnrh1 is expressed in papillary neurons, which are potential sensors for metamorphic signals, while Ci-gnrh2 is expressed in the central nervous system, including the brain vesicle, the visceral ganglion, and the nerve cord, which potentially convey and deliver metamorphic signals to the tail. Consistently, the genes for GnRH receptors are expressed in
potential targets of metamorphic signaling, including the adhesive organ, brain vesicle, muscle, notochord, and tail epidermis.

**Evolutionary Conservation of a Non-reproductive GnRH System in Chordates**

We found that *cis*-regulatory DNA of the medaka *gnrh2* gene specifically drives transcription in neurons in the hindbrain and spinal cord of medaka embryos. The expression pattern of *gnrh2*:gfp in medaka embryos is reminiscent of the expression pattern of the *gnrh* genes in the ascidian larva. We further confirmed the expression of endogenous *gnrh2* in the hindbrain and spinal cord by RT-PCR. In the hindbrain, *gnrh2*:gfp was expressed in reticulospinal neurons, which control spinal locomotor circuits [64–66]. In the spinal cord, *gnrh2*:gfp was expressed in at least two distinct types of neurons: commissural interneurons and a group of ipsilaterally projecting interneurons. Commisural interneurons provide reciprocal coordination between the left and right sides of the spinal cord as parts of central pattern generators (CPGs), the
neural networks generating rhythmic movements such as swimming and walking [63–68]. The ipsilaterally projecting interneurons expressing gnh-2::gfp have been found to be morphologically similar to the V1 spinal neurons. The V1 interneurons are inhibitory neurons that innervate motor neurons and play an important role in the locomotor CPG [45–47]. Thus, GnRH signaling may regulate the function or development of neural circuits that generate rhythmic movements in vertebrates.

In Ciona larvae, the locomotor CPG has been proposed in the motor ganglion and the anterior nerve cord [27,60], where the gnh genes are also expressed. Homology between the tunicate motor ganglion nerve cord and the vertebrate hindbrain/spinal cord has been suggested by developmental studies, such as those examining the expressions and functions of conserved transcription factors and signaling molecules [69–72]. Taken together with these previous findings, the current data suggest the deep evolutionary origin of a non-reproductive embryonic/larval GnRH system in chordates.

Materials and Methods

Ethics Statement

All the treatments of animals in this research followed the Japanese Act on Welfare and Management of Animals (Act No. 105 of October 1, 1973; the latest revisions Act No.68 of 2005, Effective June 1, 2006) and were approved by the Institutional Animal Care and Use Committee of Konan University (approval nos. K-11-04 and K-10-02). No specific permits were required for the described field studies.

Animals and Embryos

Mature adults of C. intestinalis were collected from harbors in Murotsu and Aioi, Hyogo, Japan, or obtained from the National BioResource Project (NBRP) and maintained in indoor tanks of artificial seawater (Marine Art BR, Senju Seiyaku, Osaka, Japan) at 18°C. Embryos and larvae were prepared as described previously [73].

Mature adults of the orange-red variety of medaka Oryzias latipes were a gift from Dr. Rie Kusakabe (Kobe University). They were kept in indoor tanks under artificial reproductive conditions (9 h 45 min dark, 14 h 15 min light; 28°C) and fed on Otohime B2 artificial feed (Marubeni Nishin Feed Co., Ltd., Tokyo, Japan). Naturally spawned and fertilized eggs were collected and the embryos were cultured in distilled water containing 0.6-ppm methylene blue at 28°C. Embryos were dechorionated using hatchening enzyme [74] provided by the NBRP-Medaka.

Isolation and Characterization of cDNAs Encoding C. intestinalis GnRHRs

The genes encoding Ci-GnRHR3 and Ci-GnRHR4 were found in the C. intestinalis genome database [75] by TBLASTN searches using amino acid sequences of human and C. Embryos and larvae were prepared as described previously [73].

Mature adults of the orange-red variety of medaka Oryzias latipes were a gift from Dr. Rie Kusakabe (Kobe University). They were kept in indoor tanks under artificial reproductive conditions (9 h 45 min dark, 14 h 15 min light; 28°C) and fed on Otohime B2 artificial feed (Marubeni Nishin Feed Co., Ltd., Tokyo, Japan). Naturally spawned and fertilized eggs were collected and the embryos were cultured in distilled water containing 0.6-ppm methylene blue at 28°C. Embryos were dechorionated using hatchening enzyme [74] provided by the NBRP-Medaka.

Isolation and Characterization of cDNAs Encoding C. intestinalis GnRHRs

The genes encoding Ci-GnRHR3 and Ci-GnRHR4 were found in the C. intestinalis genome database [75] by TBLASTN searches using amino acid sequences of human and C. intestinalis GnRHRs as queries. A 1231- or 751-bp cDNA fragment encoding a portion of Ci-GnRHR3 or Ci-GnRHR4 was amplified from a mid tailbud cDNA pool by PCR with a pair of primers (5’-CAACACTATT-CAACTAAGGGGAG-3’ and 5’-AGTATGGTAAAGCGGACAG-TATGTCC-3’ for Ci-GnRHR3; 5’-CTGCATTGTGGTTGACAG-TGCCTC-3’ and 5’-TAAAAGAATACCCTGATGCT-3’ for Ci-GnRHR4). The 5’ end of Ci-GnRHR3 mRNA was determined by the 5’ RACE method using a Gene Racer Kit (Invitrogen). The primer sequences used for 5’ RACE were 5’-GGCTTAAAGCCGGTACCCCTGTC-TAC-3’ (for the primary PCR) and 5’-ACCTGTAAGCACACTGATA-GTGA-3’ (for the nested PCR). The full-length coding sequence of Ci-GnRHR3 cDNA was amplified by PCR from a cDNA pool of mid tailbud embryos using gene-specific primers (forward primer 5’-GAACCTGTGATACAGGTTGTAGT-3’; primary reverse primer 5’-AGTATGGTAAAGCAAAGTTAT-TAAGTG-3’; nested reverse primer 5’-ATGGTACGCCAAC-GATG-3’). The 5’ end and 3’ end of Ci-GnRHR4 mRNA were determined by the 5’ RACE and 3’ RACE methods, respectively, using a Gene Racer kit (Invitrogen). The primer sequences used for 3’ RACE were 5’-GGCTTAAAGCCGGTACCCCTGTC-TAC-3’ (for the primary PCR) and 5’-GAAGAGGCGAACAAAGG-CAACGCAACT-3’ (for the nested PCR). The full-length coding sequence of Ci-GnRHR4 cDNA was amplified by PCR from a cDNA pool of mid tailbud embryos using gene-specific primers (forward primer 5’-AGTATGGTAAAGCGGACAG-TATGTCC-3’ and 5’-CTGATGGTAAAGCGGACAG-TATGTCC-3’ for the primary PCR) and 5’-GAAGAGGCGAACAAAGG-CAACGCAACT-3’ (for the nested PCR). The full-length coding sequence of Ci-GnRHR4 cDNA was cloned into a pBluescript II SK(+) vector. The cDNA clones were sequenced on both strands by the cycle sequencing method with an Applied Biosystems 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Molecular Phylogenetics

The protein dataset analyzed consisted of 55 receptor sequences as well as the human oxytocin and vasopressin V2 receptors, used as outgroups (Table S1). Sequences were retrieved from Uniprot, GenBank/NCBI and Joint Genome Institute (JGI) web sites. Multiple sequence alignment was performed using TM-Coffee [31]. The per-site confidence of aligned residues was assessed using the CORE index [part of the T-Coffee suite [76]]. The CORE index reflects the consistency of aligned residues with respect to the TM-Coffee alignment library; only residues with CORE index 8–9 (on a 0–9 scale) were retained. The alignment was inspected manually and edited using SeaView [77]. The final alignment (available upon request) consisted of 243 sites. ProtTest v3.2 [78] identified JTT +F1+I as the best model of protein evolution that fit the data, according to all the statistics implemented; this model was used to infer the ML trees. In order to better explore the tree-topology space, we used PhyML v3 (build 20120412) [32], Leaphy v1.0 [33] and RAxML v7.2.8 [34]. Tree-topology searches in PhyML were conducted using the Subtree Pruning and Regrafting moves (starting from 10 random trees and the BioNJ tree). Branch support values were estimated in PhyML by 100 non-parametric bootstrap replicates and by the less conservative parametric aBayes statistic (Bayesian-like transformation of approximate likelihood ratio test [79]).

As far as the position of the sea urchin receptors is concerned, we reconstructed manually the topology proposed by Roch et al. in [30], and compared it with the one obtained in the present analysis. Their statistical confidence was assessed using the SH [80], KH [81] and ELW [82] tests as implemented in TreePuzzle v5.2 [83].

Whole-mount in situ Hybridization

For synthesizing the Ci-GnRHR1 and Ci-GnRHR2 probes, the full-length cDNA clones previously reported [20] were used. For Ci-GnRHR3 and Ci-GnRHR4 probes, cDNA clones containing the full-length coding region for Ci-GnRHR3 and Ci-GnRHR4 described above were used. A Ci-gnrh1 cDNA fragment was amplified from a larval cDNA pool by PCR with a pair of gene-specific primers (5’-GAATTCACCCTGATACAGGTTGTAGT-3’ and 5’-GAACCTGTGATACAGGTTGTAGT-3’), and subcloned into pBlue-
scriptII SK[+]. The cDNA clone for Ci-gnrh2 (Gene Collection ID GC27a12) was obtained from the Ciona Genome Collection release 1 [84]. The plasmid clones were digested with a restriction enzyme, and used as the template to synthesize a digoxigenin-labeled antisense RNA probe using a DIG RNA labeling kit (Roche, and used as the template to synthesize a digoxigenin-labeled antisense RNA probe using a DIG RNA labeling kit (Roche, Germany) and used as the template to synthesize a digoxigenin-labeled RNA probe using the DIG RNA labeling kit (Roche, Germany). The cDNA of total RNA was used as the template to synthesize the first strand cDNA using an oligo(dT) primer according to the manufacturer’s protocol (SuperScript[+] III First Strand Synthesis System for RT-PCR; Invitrogen Corp., Carlsbad, CA). The cDNA fragment of gnrh1, gnrh2, gnrh3 or cytoskeletal actin OICa1 [42], which was used as a control, was amplified by PCR from the first-strand cDNA. The primer sets used were as follows: for gnrh1, 5'-ACTTATGAACCT-GAAGTCTGTTG-3' and 5'-TTACACTCCCAA-GAAAGCC-3'; for gnrh2, 5'-ACCACTGAGTAAAGTGTC-3' and 5'-CAAGGGATCCAGAAGAATATTTCTC-3'; for gnrh3, 5'-GAAAACACAGAGTTCTAATGGACG-3' and 5'-GAGCCATCATTAATAGGTTGTAATGTC-3'; and for OICa1, 5'-GAACGGATCTGTTG-3' and 5'-GACTTCCATACCAAGGAGAA-3'. After denaturation at 96°C for 2 min, the PCR was performed for 35 cycles at 98°C for 10 sec, 55°C for 30 sec, and 72°C for 1 min followed by a final extension at 72°C for 7 min using 5% (1.0 µl) of the cDNA reaction mixture as a template, 25 pmol of each primer, and 1.25 U of a Taq DNA polymerase (Ex Taq; Takara Bio, Japan). To eliminate the possibility of PCR amplification derived from contaminated genomic DNA, 35 cycles of amplification were carried out without reverse transcriptase as a control experiment. The PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide.

Labeling Cells with Green Fluorescent Protein (GFP)

Using cis-regulatory Regions of gnrh or GnRHR Genes

The 5' flanking sequences of Ci-gnrh1, Ci-gnrh2, Ci-GnRHR1, Ci-GnRHR2, and Ci-GnRHR3 from the JGI Genome Database were used to design gene-specific primers. Genomic DNA fragments containing an upstream region of each gene were amplified from sperm DNA by PCR using a thermostable DNA polymerase (LA Taq, Takara BIO, Japan) and a pair of gene-specific oligonucleotide primers (5'-CAAGGACCTGCTCGATCCA-CACG and 5'-CACACTGAGTATTGGTGAAGTTGAGATAAGGCA-3 for Ci-gnrh1, 5'-TGACGTGCAGAGGCACGATCTAATAAG-3' and 5'-ACGGATCTTGTCATGTTTCTTCTGTAAAG-3' for Ci-gnrh2, 5'-CAAGGACGTGCGTATGTGTTGAAGTTGAGATAAGGCA-3' and 5'-TGACGTGCAGAGGCTCACTGCTGCAGTTC-3 for Ci-GnRHR1, 5'-CAAGGACGTCCGTCGAACGGGAACATCATTA-3' and 5'-CAACCCGGGCTATACGACTCA-GATCGTCGTC-3 for Ci-GnRHR2, and 5'-CAAGGACCTGCTCGATCCA-CACG and 5'-CACACTGAGTATTGGTGAAGTTGAGATAAGGCA-3' for Ci-GnRHR3). The amplified upstream sequences were inserted into the SaI/Smal (Ci-gnrh1, Ci-GnRHR2, and Ci-GnRHR3), Sall/BamHI (Ci-gnrh2), or SaI/Sall (Ci-GnRHR1) sites of pBluescript-EGFP [86]. The sizes of the genomic DNA fragments inserted were 4.7 kb (Ci-gnrh1), 4.3 kb (Ci-gnrh2), 5.1 kb (Ci-GnRHR1), 4.6 kb (Ci-GnRHR2), and 5.7 kb (Ci-GnRHR3). The promoter-GFP fusion construct was electroporated into fertilized eggs of C. intestinalis as described by Corbo et al. [87]. The reporter expression was observed as described previously [36]. The 5' flanking sequence of the medaka gnrh2 gene obtained from the Ensembl genome database was used to design gene-specific primers. The O. latipes genomic DNA was extracted from one individual of the Hsd-r+ inbred strain as described previously [43]. Genomic DNA fragments containing an upstream region of gnrh2 were amplified from the genomic DNA by PCR using a thermostable DNA polymerase (PrimeStar HS DNA polymerase, Takara BIO, Japan) and a pair of gene-specific oligonucleotide primers, 5'-CAAACGATCGTTC-3 and 5'-TAATGTC-3 for Ci-gnrh1, 5'-CAACCCGGGATTGTCGAATATGTGGTAGTCAGCAA-3 and 5'-CAACCCGGGCTATACGACTCA-GATCGTCGTC-3 for Ci-GnRHR1, 5'-TGACGTGCAGAGGCTCACTGCTGCAGTTC-3 for Ci-GnRHR2, and 5'-CAAGGACCTGCTCGATCCA-CACG and 5'-CACACTGAGTATTGGTGAAGTTGAGATAAGGCA-3' for Ci-GnRHR3). The amplified upstream sequences were inserted into the SaI/Smal (Ci-gnrh1, Ci-GnRHR2, and Ci-GnRHR3), SaI/BamHI (Ci-gnrh2), or SaI/Sall (Ci-GnRHR1) sites of pBluescript-EGFP [86]. The sizes of the genomic DNA fragments inserted were 4.7 kb (Ci-gnrh1), 4.3 kb (Ci-gnrh2), 5.1 kb (Ci-GnRHR1), 4.6 kb (Ci-GnRHR2), and 5.7 kb (Ci-GnRHR3). The promoter-GFP fusion construct was electroporated into fertilized eggs of C. intestinalis as described by Corbo et al. [87]. The reporter expression was observed as described previously [36].

The full-length open reading frame of each Ci-GnRHR cDNA was cloned into a pcDNA3.1 plasmid vector. COS-7 cells and HEK293-MSR cells were purchased from American Type Culture Collection (ATCC; Rockville, MD, U.S.A.) and Invitrogen Japan (Tokyo, Japan), respectively. COS-7 cells or HEK293-MSR cells were grown under 5% CO2 and 100% relative humidity in DMEM medium supplemented with 10% (v/v) fetal bovine serum and 0.1 mM non-essential amino acids. Each Ci-GnRHR open reading frame-containing pcDNA 3.1 plasmid or empty vector was transfected into COS-7 cells using Lipofectamine 2000 (Invitrogen, San Diego, CA, U.S.A) or HEK293-MSR cells using Targefect F-1 reagent (Targeting Systems, San Diego, CA, U.S.A.) according to the manufacturer’s instructions, and the cells were incubated for 24 hours (inositol phosphate assay) or 48 hours (measurement of intracellular Ca2+ and cAMP).

Inositol Phosphate Assay

Twenty-four hours after transfection, the COS-7 cells were washed twice with Medium 199 (Nacalai Tesque, Kyoto, Japan) and incubated in Medium 199 containing 2% (vol/vol) dialyzed FCS and labeled with 1 µCi [3H]myo-inositol with PT6-271 stabilizer (TRK-912, Amersham) per well at 37°C for 48 hours. Medium was then removed, and cells were washed twice with buffer A (140 mM NaCl, 20 mM Hepes, 4 mM KCl, 0 mM D-glucose, 1 mM MgCl2, 1 mM CaCl2). Then cells were preincubated with buffer A containing 10 mM LiCl for 15 min, followed by addition of the GnRHs at various concentrations at 37°C for 45 min. The reaction was stopped by removing the incubation medium and adding 1 ml of ice-cold 0.6 M perchloric acid and 0.2 mg/ml phytic acid. The samples were then transferred to microcentrifuge tubes, kept on ice for 10 min, and then neutralized by adding 350 µl of 1 M K2CO3/5 mM EDTA. After neutralization, the samples were centrifuged at 10,000 × g at 4°C for 2 min, and the supernatants were transferred to columns containing AG 1-X8 anion-exchange resin (Bio-Rad). Total inositol phosphates were eluted with 1.05 M ammonium formate/0.1 M formic acid, and radioactivity was determined.

Determination of Intracellular Calcium Ion and cAMP Levels

For measurement of ligand-induced intracellular calcium ion, HEK293-MSR cells were loaded for 60 min with Ca3 fluorescent calcium indicator mixture (Molecular Devices, Sunnyvale, CA, USA).
U.S.A) 48 hours after transfection. Each t-GnRH ligand at various concentrations was automatically administrated to the cells in a FlexStation II apparatus (Molecular Devices). Real-time fluorescence kinetics at an excitation wavelength of 485 nm and emission wavelength of 525 nm was observed for 3 minutes using a FlexStation II apparatus. Cyclic AMP production was evaluated by a fluorescent competitive immunoassay for cAMP using CatchPointTM Cyclic AMP assay kit (Molecular Devices) and FlexStation II according to the manufacturer’s instructions.

**Supporting Information**

**Figure S1** GnRH peptides and gnrh genes in Ciona intestinalis. (A) Structure of the two C. intestinalis genes encoding GnRHs. 5’ is to the left. White boxes indicate untranslated regions (noncoding exons sequences), black boxes indicate coding sequences, and each of the gray boxes with a number (3, 4, 5, 6, 7 or 8) indicates the region encoding a single GnRH peptide shown in (B). Horizontal lines indicate introns and intergenic sequences. The gene model ID for each gene is indicated in parentheses. (B) Primary structure of six C. intestinalis GnRHRs. “pGlu” refers to the N-terminal pyroglutamic acid, and “Gly-NH2” indicates the C-terminal glycine amide. This figure is modified from [19].

**(TIF)**

**Figure S2** Amino acid comparison among Ci-GnRHRs. The deduced amino acid sequences of Ci-GnRHR1, Ci-GnRHR2, Ci-GnRHR3, and Ci-GnRHR4 from a Pacific population (Pac; this study) were aligned with those from an Atlantic population (Atl) [21] using the ClustalW program [80], and the alignment was optimized manually. Dashes indicate gaps introduced in the sequence to optimize the alignment. Amino acid residues showing polymorphism between Pacific and Atlantic sequences are indicated in red. The putative transmembrane domains (TM1-TM7) are indicated by horizontal lines. Triangles indicate positions of introns in the gene encoding each Ci-GnRHR.

**(TIF)**

**Figure S3** Molecular phylogeny of the GnRHR family. Maximum-likelihood (ML) trees were computed by using PhyML v3 (build 20120412), Leaphy v1.0 and RAxML v7.2.8, from a dataset comprising 57 taxa (243 sites). Human oxytocin (OXYR) and vasopressin V2 receptors (V2R) are used as outgroups. The evolutionary model was JTT+F+I+F. Branch support values are ML non-parametric bootstrap replicates/parametric aBayes. Only bootstrap values ≥75%, and aBayes values ≥0.95 are shown.

Scale bar indicates the estimated number of substitutions per site. Tree topology is from Leaphy. *It* was not possible to decide which one of GnRHR3 or GnRHR4 is more closely related to the GnRHR1/GnRHR2 clade (no statistical support for either topology).

**(TIF)**

**Figure S4** Signaling activities of various tGnRHs at Ci-GnRHRs expressed in HEK293-MSR cells. (A) Elevation of intracellular calcium ion mediated by Ci-GnRHR1 in response to tGnRHs. (B) cAMP production mediated by Ci-GnRHR1. (C) cAMP production mediated by Ci-GnRHR-2. (D) cAMP production mediated by Ci-GnRHR3. (E) Elevation of intracellular calcium ion in the cells co-expressing Ci-GnRHR1 and Ci-GnRHR4 in response to tGnRHs. (F) cAMP production in the cells co-expressing Ci-GnRHR1 and Ci-GnRHR4. (G) cAMP production in the cells co-expressing Ci-GnRHR-1 and Ci-GnRHR3. Data represent means ± S.E.M. of two independent experiments.

**(TIF)**

**Table S1** List of proteins with their accession numbers used for the phylogenetic analysis.

**(XLS)**

**Table S2** EC50 values (nM) of tGnRHs for inositol phosphate accumulation in COS-7 cells expressing Ci-GnRHR1.

**(DOC)**

**Acknowledgments**

We thank Drs. Yasunori Murakami, Shin-ichi Higashijima, Yasunori Sasakura, and Takeo Horie for valuable discussion; Drs. Nori Satoh and Yutaka Satou for the Ciona gene collection plates; National BioResource Project (NBRP) of MEXT, and all members of the Maizuru Fisheries Research Station of Kyoto University and Misaki Marine Biological Station of the University of Tokyo for providing us with C. intestinalis adults; NBRP-Medaka for providing us with medaka hatching enzyme; and the Murotsu Fisheries Cooperative and fishermen at Murotsu Port for generous use of the fishery facility.

**Author Contributions**

Conceived and designed the experiments: TGK TS YK TT YD YM TT YD GM HY. Performed the experiments: TGK TS MA YK YM TT YD KF HS MT. Contributed reagents/materials/analysis tools: TGK GM HY MKP HS MT. Performed the experiments: TGK TS MA YK YM TT YD KF YT GM YS HY MKP HS. Analyzed the data: TGK TS YK GM HS MT. Contributed reagents/materials/analysis tools: TGK GM HY MKP HS MT. Wrote the paper: TGK HS MT.

References

1. Yamamoto N (2003) Three gonadotropin-releasing hormone neuronal groups with special reference to teleosts. Anat Sci Int 78: 139–153.
2. Okubo K, Nagahama Y (2008) Structural and functional evolution of gonadotropin-releasing hormone in vertebrates. Acta Physiol 193: 3–15.
3. Oka Y (2009) Three types of gonadotropin-releasing hormone neurones and steroid-sensitive sexually dimorphic kispeptin neurones in teleosts. J Neuroendocrinol 21: 314–338.
4. Kanda S, Nishikawa K, Karigo T, Okubo K, Isomae S, et al. (2010) Regular pacemaker activity characterizes gonadotropin-releasing hormone 2 neurons recorded from green fluorescent protein-transgenic medaka. Endocrinology 151: 695–701.
5. Dolan S, Evans NP, Richter TA, Nolan AM (2003) Expression of gonadotropin-releasing hormone and gonadotropin-releasing hormone receptor in sheep spinal cord. Neurons Lett 346: 120–122.
6. Chen CC, Fernando RD (2006) Distribution of two gonadotropin-releasing hormone receptor types in a cichlid fish suggest functional specialization. J Comp Neurol 495: 314–323.
7. Flanagan CA, Chen CC, Coetser M, Mamputha S, Whitlock KE, et al. (2007) Expression, structure, function, and evolution of gonadotropin-releasing hormone (GnRH) receptors GnRH-R1SHS and GnRH-R2PEY in the teleost, Astatotilapia burtoni. Endocrinology 148: 5060–5071.
8. Albertson AJ, Talbott H, Wang Q, Jensen D, Skinner DC (2008) The gonadotropin-releasing hormone type I receptor is expressed in the mouse cerebellum. Cerebellum 7: 379–384.
9. Yamamoto N, Oka Y, Amano M, Aida K, Haegawa Y, et al. (1993) Multiple gonadotropin-releasing hormone (GnRH)-immunoreactive systems in the brain of the dwarf gourami, Colisa laliah: immunohistochemistry and radioimmunoassay. J Comp Neurosci 35: 353–368.
10. Sherwood N, Wu S (2005) Developmental role of GnRH and PACAP in a zebrafish model. Gen Comp Endocr 42: 74–80.
11. Wu S, Page I, Sherwood NM (2006) A role for GnRH in early brain regionalization and eye development in zebrafish. Mol Cell Endocrinol 257–258: 47–64.
12. Abraham E, Palevitch O, Ijiri S, Du SJ, Gothilf Y, et al. (2008) Early development of forebrain gonadotropin-releasing hormone (GnRH) neurons and the role of GnrH as an autocrine migration factor. J Neuroendocrinol 20: 394–405.
13. Kanaho YI, Enomoto M, Endo D, Machiro S, Park MK, et al. (2008) Neurotrophic effect of gonadotropin-releasing hormone on neurite extension and neuronal migration of embryonic gonadotropin-releasing hormone neurons in chick olfactory nerve bundle culture. J Neurosci Res 67: 2257–2264.

14. Ramakrishnan S, Lee W, Navarre S, Kodolicky DJ, Wayne NL (2010) Acquisition of spontaneous electrical activity during embryonic development of gonadotropin-releasing hormone-3 neurons located in the terminal nerve of transgenic zebrafish (Danio rerio). Gen Comp Endocrinol 168: 401–407.

15. Deuticke F, Behzmann H, Chouartres D, Philippi H (2006) Tnntacutes and not cephalephorines are the closest living relatives of vertebrates. Nature 439: 965–968.

16. Putnam NH, Butts T, Ferrier DE, Loring RF, Holland U, et al. (2008) The amphioxus genome and the evolution of the chordate karyotype. Nature 453: 1064–1071.

17. Okihuma M, Katagiri Y, Nakagawa M, Tsuda M (2000) Possible involvement of light regulated gonadotropin-releasing hormone neurons in biological clock for reproduction in the cerebral ganglion of the ascidian, Halocynthia roretzi. Neurosci Lett 293: 5–8.

18. Terakado K (2001) Induction of gamete by gonadotropin-releasing hormone in a postchordate, Ciona intestinalis. Gen Comp Endocrinol 122: 277–288.

19. Adams BA, Tello JA, Erchechi J, Warby C, Hong DJ, et al. (2003) Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protochordate, Ciona intestinalis. Endocrinology 144: 1967–1973.

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

21. Tello JA, Rivier JE, Sherwood NM (2005) Tunicate gonadotropin-releasing hormone (GnRH) peptides selectively activate gonadotropin-releasing hormone receptors found in the neural complex of the ascidian, Ciona intestinalis. Gen Comp Endocrinol 141: 426–432.

22. Makie GO, Wyeth RC (2000) Conduction and coordination in deganglionated ascidians. Can J Zool 78: 1626–1639.

23. Makie GO, Sakai CL (2000) Cephalochordates: organ systems in the presumptive ancestral chordate, Ciona intestinalis (Ascidian). Gen Comp Endocrinol 112: 426–434.

24. Powell JFF, Reska-Skinner SM, Prakash MO, Fischer WH, Park M, et al. (1996) The ascidian Ciona intestinalis: a novel EGF-like protein, plays a central role in ascidian metamorphosis. Dev Growth Differ 38: 541–554.

25. Tsuda M, Sakurai D, Goda M (2003) Delineating metamorphic pathways in the ascidian Ciona intestinalis. Dev Biol 256: 357–367.

26. Chiba SA, Sakai A, Nakayama A, Takamura K, Satoh N (2004) Development of the GnRH system in chordates. Dev Growth Diff. 46: 1–13.

27. Nishino A, Okamura Y, Piscopo S, Brown ER (2010) A glycine receptor is required for development of the GnRH system in the medaka, Oryzias latipes: evolutionary and functional implications. Gene 414: 121–131.

28. Millar RP, Lu ZL, Pashon AJ, Flanagan CA, Morgan K, Maddrey SR (2004) GnRH-gonadotropin-releasing hormone receptors. Endocr Rev 25: 235–273.

29. Ramakrishnan S, Lee W, Navarre S, Kozlowski DJ, Wayne NL (2010) Glycine receptors selectively activate gonadotropin-releasing hormone neuronal development: insights from transgenic medaka. Neurosci Biobehav Rev 34: 541–544.

30. Ovlko K, Sakai F, Lai EL, Yoshiyaki G, Takeuchi Y, et al. (2006) Forebrain gonadotropin-releasing hormone neuronal development: insights from transgenic medaka. Neurosci Biobehav Rev 30: 590–596.

31. Roach NM, Busby ER, Sherwood NM (2011) Amphioxus: beginning of vertebrate evolution and the relationship to the Xenollampallama syndrome. Endocrinology 147: 1076–1084.

32. Ramakrishnan S, Kajii K, Iwashita Y, Watanabe Y, et al. (2003) Urochordate forebrain GnRH receptor subtypes required for skeletal and cardiac muscle-specific gene expression. Int J Dev Biol 43: 541–544.

33. Horie T, Suzuki N (2000) Photoreceptors and olfactory cells express the same retinal glycine cyclase, isom in medaka: visualization by promoter transgenics. FEBS Lett 483: 143–148.

34. Stamatakis A (2006) RAxML-VI-HPC: Maximum Likelihood-based phylogenetic analysis with thousands of taxa and mixed models. Bioinformatics 22: 2684–2688.

35. Hall RA, Swenson EM, Yoder AD, Thelen DJ (2003) RAxML-v1.0: Maximum Likelihood-based phylogenetic analysis using fast bootstrap computation. Systematic Biology 52: 406–414.

36. grillner S, Parker D, el Manira A (1998) Vertebrate locomotion—a lamprey model. Physiol Rev 78: 1041–1046.

37. Dong B, Horie T, Denker E, Kusakabe T, Tsuda M, et al. (2009) Tube neuron of retinal ganglion cell in medaka: Delineating metamorphic pathways in the ascidian Ciona intestinalis. Dev Biol 326: 357–367.

38. Horie T, Kusakabe T, Suzuki N, Satake H (2010) Functional diversity of signaling pathways through G protein-coupled receptor heterodimerization with a species-specific orphan receptor subtype. Mol Biol Evol 27: 1106–1110.

39. Horie T, Asakawa I, Aoyama M, Kusakabe T, Tsuda M, et al. (2008) Delineating metamorphic pathways in the ascidian Ciona intestinalis. Proc Natl Acad Sci U S A 105: 10461–10463.

40. Ikuta T, Saiga H (2007) Dynamic change in the expression of developmental gene 322: 77–84.

41. Okubo K, Nagata S, Ko R, Kataoka H, Yoshiura Y, et al. (2001) Identification and characterization of two distinct GnRH receptor subtypes in a teleost, the medaka Oryzias latipes. Endocrinology 142: 4729–4739.

42. Horie T, Suzuki N (2000) Photoreceptors and olfactory cells express the same retinal glycine cyclase, isom in medaka: visualization by promoter transgenics. FEBS Lett 483: 143–148.

43. Carrette F, Beintzmann H, Chouartres D, Philippi H (2006) Tnntacutes and not cephalephorines are the closest living relatives of vertebrates. Nature 439: 965–968.

44. Makie GO, Wyeth RC (2000) Conduction and coordination in deganglionated ascidians. Can J Zool 78: 1626–1639.

45. Cheng J-M, Di Tommaso P, Taly J-F, Notredame C (2012) Accurate multiple sequence alignment of transmembrane proteins with PSI-Coffee. BMC Bioinformatics 13 (Suppl 4): S1.

46. Guindon S, Gascuel O (2003) PhyML: A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696–704.

47. Whelan S (2007) New approaches to phylogenetic tree search and their application to large numbers of protein alignments. Syst Biol 56: 722–740.

48. Stamatakis A (2006) RaxML-VI-HPC: Maximum Likelihood-based phylogeny analyses with thousands of taxa and mixed models. Bioinformatics 22: 2684–2688.

49. Tello JA, Sherwood NM (2003) Aminophosph: beginning of vertebrate and end of invertebrate type GnRH receptor lineage. Gen Comp Endocrinol 136: 11–18.

50. Makie GO, Wyeth RC (2000) Conduction and coordination in deganglionated ascidians. Can J Zool 78: 1626–1639.

51. Horie T, Kusakabe T, Suzuki N, Satake H (2010) Functional diversity of signaling pathways through G protein-coupled receptor heterodimerization with a species-specific orphan receptor subtype. Mol Biol Evol 27: 1106–1110.

52. Horie T, Asakawa I, Aoyama M, Kusakabe T, Tsuda M, et al. (2008) Delineating metamorphic pathways in the ascidian Ciona intestinalis. Dev Biol 312: 631–643.
72. Stolfi A, Levine M (2011) Neuronal subtype specification in the spinal cord of a protovertebrate. Development 138: 995–1004.
73. Nakagawa M, Miyamoto T, Ohkuma M, Tsuda M (1999) Action spectrum for the photic response of Ciona intestinalis (Ascidacea, Urochordata) larvae implicates retinal proteins. Photosynth Res 67: 339–362.
74. Yamagami K (1972) Isolation of a choriolytic enzyme (hatching enzyme) of the teleost, Oryzias latipes. Dev Biol 29: 343–348.
75. Dehal P, Satou Y, Campbell RK, Chapman J, Degnan B, et al. (2002) The draft genome of Ciona intestinalis: insights into chordate and vertebrate origins. Science 298: 2157–2167.
76. Notoedame C, Higgins D, Heringa J (2000) T-Coffee: A novel method for multiple sequence alignments. J Mol Biol 302: 205–217.
77. Gouy M, Guindon S, Gascuel O (2010) SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol Biol Evol 27: 1164–1165.
78. Darriba D, Taboada GL, Doallo R, Posada D (2011) ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics 27: 1164–1165.
79. Anisimova M, Gil M, Dufayard JF, Dessimoz C, Gascuel O (2011) Survey of branch support methods demonstrates accuracy, power, and robustness of fast likelihood-based approximation schemes. Syst Biol 60: 685–99.
80. Shimodaira H, Hasegawa M (1999) Multiple comparisons of loglikelihoods with applications to phylogenetic inference. Mol Biol Evol 16: 1114–1116.
81. Kishino H, Hasegawa M (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominioidea. J Mol Evol 29: 170–179.
82. Strimmer K, Rambaut A (2002) Inferring confidence sets of possibly misspecified gene trees. Proc R Soc Lond B 269: 137–142.
83. Schmidt HA, Strimmer K, Vingron M, von Haeseler A (2002) TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics 18: 502–504.
84. Satou Y, Yamada L, Mochizuki Y, Takatori N, Kawashima T, et al. (2002) A cDNA resource from the basal chordate Ciona intestinalis. Genesis 33: 153–154.
85. Nakashima Y, Kusakabe T, Kusakabe R, Terakita A, Shichida Y, et al. (2003) Origin of the vertebrate visual cycle: genes encoding retinal photosisomerase and two putative visual cycle proteins are expressed in whole brain of a primitive chordate. J Comp Neuro 460: 180–190.
86. Yoshida R, Sakurai D, Horie T, Kawakami I, Tsuda M, et al. (2004) Identification of neuron-specific promoters in Ciona intestinalis. Genesis 39: 130–140.
87. Corbo JC, Levine M, Zeller RW (1997) Characterization of a notochord-specific enhancer from the Brachyury promoter region of the ascidian, Ciona intestinalis. Development 124: 509–602.
88. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680.