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General and Comparative Endocrinology 141 (2005) 76–83
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

Distribution of gonadotropin-releasing hormone (GnRH) by in situ hybridization in the tunicate *Ciona intestinalis*

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Received 25 June 2004; revised 28 November 2004; accepted 29 November 2004
Available online 8 January 2005

Abstract

Gonadotropin releasing hormone (GnRH) is the key hypothalamic neurohormone that is critical in its role of reproduction in all vertebrates. There are currently twenty-four known forms of GnRH that have been identified, 14 in vertebrates and 10 in invertebrates. In tunicates, the primary structure of nine forms have been identified, all of which have been shown to stimulate gamete release. However, the distribution and function of the various GnRH peptides in tunicates have not been fully examined. Therefore, the objective of this study was to determine tissue specific expression of *Ci-gnrh-1* and *Ci-gnrh-2* in an adult tunicate, *Ciona intestinalis*, using reverse transcriptase-polymerase chain reaction (RT-PCR) and in situ hybridization. To examine the expression of the two GnRH genes, total RNA and genomic DNA were isolated from whole animals. Total RNA from neural tissue (cerebral ganglion and neural gland), testis, ovary, heart, and hepatic organ were also isolated. Results from RT-PCR indicated both forms are only expressed in the neural tissue. We extended these studies using fluorescent dual label in situ hybridization. GnRH expression was confirmed to be in the cerebral ganglion bordering the neural gland. These current data along with previous studies suggest that GnRH may be involved in reproduction in the protochordate.

Keywords: Gonadotropin-releasing hormone; GnRH; Tunicate; Ganglion; *Ciona intestinalis*

1. Introduction

Gonadotropin releasing hormone (GnRH) is an essential hypothalamic neurohormone which is responsible for the development and maintenance of reproductive systems in all vertebrates. Currently there are 24 isoforms of GnRH which have been identified by either protein purification or cDNA isolation, 14 distinct GnRH isoforms in vertebrates and 10 in invertebrates (Table 1). Nine of the 10 invertebrate GnRHs have been identified in three species of tunicate (tunicate GnRH): *Chelyosoma productum* (tunicate GnRH-1 and -2, tGnRH-I and -II), *Ciona intestinalis* (tGnRH-III to -VIII), and *C. savignyi* (tGnRH-IX) (Adams et al., 2003; Powell et al., 1996). In all taxa in which GnRH has been investigated, the phylum Tunicata (Urochordata) represents one of the most ancient lineages of chordates. Within this group *C. intestinalis* has become an excellent model for studying and comparing reproductive control systems to the vertebrate lineage, especially since a draft of the *C. intestinalis* genome was completed in 2002 which allows for in-depth molecular comparisons with other chordates (Dehal et al., 2002).

The structures of the nine tunicate GnRH's have been identified by protein purification (tGnRH-I and -II) and in silico analysis followed by cDNA isolation (tunicate GnRH-III to -IX) (Adams et al., 2003; Powell et al., 1996). The question arises to whether each of these GnRHs are involved in mediating reproduction in

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doi:10.1016/j.ygcen.2004.11.012

Table 1
GnRH family of peptides

	1			2	3	4	5	6	7	8	9	10
Vertebrate												
Mammal	pGlu			His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	GlyNH2
Guinea Pig	pGlu			Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	GlyNH2
Chicken-I	pGlu			His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	GlyNH2
Rana	pGlu			His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	GlyNH2
Seabream	pGlu			His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	GlyNH2
Salmon	pGlu			His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	GlyNH2
Medaka	pGlu			His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	GlyNH2
Catfish	pGlu			His	Trp	Ser	His	Gly	Leu	Asn	Pro	GlyNH2
Herring	pGlu			His	Trp	Ser	His	Gly	Leu	Ser	Pro	GlyNH2
Chicken-II	pGlu			His	Trp	Ser	His	Gly	Trp	Tyr	Pro	GlyNH2
Dogfish	pGlu			His	Trp	Ser	His	Gly	Trp	Leu	Pro	GlyNH2
Lamprey-III	pGlu			His	Trp	Ser	His	Asp	Trp	Lys	Pro	GlyNH2
Lamprey-I	pGlu			His	Trp	Ser	Leu	Glu	Trp	Lys	Pro	GlyNH2
Whitefish	pGlu			His	Trp	Ser	Tyr	Gly	Met	Asn	Pro	GlyNH2
Invertebrate												
Octopus	pGlu	Asn	Tyr	His	Phe	Ser	Asn	Gly	Trp	His	Pro	GlyNH2
Tunicate-I	pGlu			His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	GlyNH2
Tunicate-II	pGlu			His	Trp	Ser	Leu	Cys	His	Ala	Pro	GlyNH2
Tunicate-III	pGlu			His	Trp	Ser	Tyr	Glu	Phe	Met	Pro	GlyNH2
Tunicate-IV	pGlu			His	Trp	Ser	Asn	Gln	Leu	Thr	Pro	GlyNH2
Tunicate-V	pGlu			His	Trp	Ser	Tyr	Glu	Tyr	Met	Pro	GlyNH2
Tunicate-VI	pGlu			His	Trp	Ser	Lys	Gly	Tyr	Ser	Pro	GlyNH2
Tunicate-VII	pGlu			His	Trp	Ser	Tyr	Ala	Leu	Ser	Pro	GlyNH2
Tunicate-VIII	pGlu			His	Trp	Ser	Leu	Ala	Leu	Ser	Pro	GlyNH2
Tunicate-IX	pGlu			His	Trp	Ser	Asn	Lys	Leu	Ala	Pro	GlyNH2

The family of the twenty-four identified GnRH isoforms, 14 GnRHs in vertebrates, and 10 GnRHs in invertebrates (Gorbman and Sower, 2003).

a protochordate. Even though the major function of each of the nine GnRHs is unknown, studies have shown that these GnRHs may be involved in reproductive activities in tunicates. Synthetic forms of the tunicate GnRHs (tGnRH-II through -IX) were shown to stimulate gamete release in *Ciona* (Adams et al., 2003). In earlier studies, using antisera to lamprey GnRH and tunicate GnRH-I (tGnRH-I), immunoreactive(ir)-GnRH was identified close to the gonoducts (Powell et al., 1996). These authors suggested that tGnRH-II may be released into the bloodstream and act directly on the gonads (Powell et al., 1996). Also, the injection of tGnRH-I or -II into various sites of mature *Ciona* induced gamete release (Terakado, 2001). These authors suggested that tGnRH(s) may act either directly on the gonoducts or may function as a neuromodulator of other neurons innervating the gonoducts to induce spawning. GnRH immunoreactivity was identified in the neural tissue and along the dorsal strand using antiserum to tGnRH-I (Mackie, 1995). The expression of *Ci-gnrh1* and *Ci-gnrh2* was shown by PCR to occur throughout development in *Ciona*. (Adams et al., 2003). With the recent identification of the tunicate GnRHs (nine total), further studies on the distribution and function of the various GnRHs in *C. intestinalis* can now be examined.

In previous studies, there were several reports of ir-GnRH or a GnRH-like peptide shown in tunicates by molecular, chromatographical, and immunological methods during the last twenty-four years using heterologous probes and antisera. As examples of some of these studies, using antiserum to mammalian GnRH, ir-GnRH neurons in *C. intestinalis* were initially shown to be located between the cerebral ganglion and neural gland (Georges and Dubois, 1980). Kelsall et al. (1990) reported ir-GnRH using antiserum to lamprey GnRH (Bla-5) in the cerebral ganglion and nerve roots in *Chelysoma productum*. Using the same antiserum to lamprey GnRH, other investigators using serial sections of the cerebral ganglion identified ir-GnRH neurons both in normal and in adult *C. intestinalis* after cerebral ganglion regeneration had occurred suggesting a possible role of GnRH neuroblasts in the regeneration of the neural complex (Bollner et al., 1997).

In light of these various studies using either homologus or heterologous probes, it is clear that there is a distinct distribution of the GnRHs in and around the brain and central nervous system in the tunicate using immunocytochemistry, HPLC, RIA, electron microscopy, and PCR. To date there have been no studies reported examining the expression of GnRH by in situ hybridization. Therefore, the objective of this study was to determine tissue specific RNA expression of *Ci-gnrh1* and *Ci-gnrh2* in adult *C. intestinalis* using reverse transcriptase PCR (RT-PCR) and dual-label fluorescent

in situ hybridization. This method has the advantage of distinguishing the two cDNAs of GnRH.

2. Materials and methods

2.1. Animals

Adult *C. intestinalis* (103) were collected from floats maintained by Dr. Larry Harris of the University of New Hampshire (UNH) at the UNH's Coastal Marine Lab in New Castle, New Hampshire. Animals were transported to UNH in aerated sea water. Immediately, after transport to UNH, the tunic was removed and the hepatic organ, testis, ovary, heart, and neural tissue were dissected from each tunicate. The tissues were flash frozen in liquid nitrogen, placed on dry ice, and then stored at −80 °C. Additionally, *C. intestinalis* were held in aerated seawater at 4 °C for 1 week at which time the neural tissue consisting of the cerebral and neural gland was prepared for in situ hybridization.

2.2. Isolation of total RNA and synthesis of cDNA

Total RNA was isolated using the Tri-Reagent—RNA, DNA protein isolation reagent following the manufacturer's protocol (Molecular Research Center, Cincinnati, OH). First-strand cDNA synthesis was accomplished using a first-strand cDNA synthesis kit from Amersham–Pharmacia Biotech (Amersham–Pharmacia Biotech, Amersham–Pharmacia Biotech UK. Amersham Place, Little Chalfont, Buckinghamshire, England HP7 9NA). Five micrograms of total RNA was reverse transcribed in a 33-μl reaction as follows: 15.2 μl RNase-free water was added to 4.8 μl of 1.036 μg/μl total RNA for a final concentration of 5 μg/20 μl. The solution was heated to 65 °C for 10 min then chilled on ice for 5 min. Eleven μl of bulk first-strand reaction mix was added (cloned, FPLCpure^{tm} murine reverse transcriptase, RNAgaurd^{m} (porcine), Rnase/Dnase-free BSA, dATP, dCTP, dGTP, and dTTP in aqueous buffer), followed by the addition of 1 μl of 200 μm DTT solution, and 1 μl of 0.2 μg/μl *Not*I-d(T)$_{18}$ bifunctional primer (5′-AAC TGG AAG AAT TCG CGG CCG CAG GAA T_{18}-3′). The heat denatured RNA was added to the above mixture and incubated at 37 °C for 1 h. Following incubation the first-strand cDNA was heated to 90 °C for 5 min to denature the RNA–cDNA duplex and inactivate the reverse transcriptase.

2.3. PCR of cDNA

Polymerase chain reaction of cDNA template was done to obtain appropriate size templates for RT-PCR and in situ hybridization probe synthesis. Using sequences for *Ci-gnrh1* Accession No. AY186743 (Kavanaugh and Sower) and *Ci-gnrh2* by Adams et al. (2003) accession number AY204708, primers were designed using Primer 3 web based software (Table 2). The primers were designed to include coding regions of the signal peptide, the three GnRH's, intervening sequences (*Ci-gnrh1*), processing sites, and the GnRH associated peptide (Fig. 1). Primers were designed to troponin, a structural protein, chosen as a control. Reactions were performed using a Perkin–Elmer 480 Thermal Cycler under the following conditions: activation step of 94 °C for 7 min; 94 °C for 1 min, primer specific annealing temperature 1 min, 72 °C for 1 min, and a 72 °C extension for 10 min followed with 4 °C hold.

2.4. Reverse transcriptase PCR (RT-PCR)

RNA extracts from all tissues collected were treated with RQ1-RNase free-DNase (Promega). Fifty nanogram of DNase free RNA was used in each reaction using the AccessQuick RT-PCR System (Promega) with a 0.1 μM final primer concentration in a 25.5 μL final reaction volume. The primers used were designed to amplify an approximately 400–500 bp product (Table 2). Thermal cycling was performed using an Eppendorf

Table 2
Primers, primer sequence, and direction

Primer name	Primer sequence	Direction
AP1	5′-CCATCCTAATACGACTCACTATAGGGC-3′	F
AP2	5′-ACTCACTATAGGGCTCGAGCGGC-3′	R
NOT1	5′-AACTGGAAGAATTCGCGGCCGCAGGAA(T)18	F
CI8F	5′-AACTTGCGGCTTTGTTGCAGTCGAGA-3′	F
CI24-1F	5′-CTCGATCTTGGTTTAGGAGTCG-3′	F
CI25-1R	5′-CATTCCTAGGTCTCGGTTGTT-3′	R
CI22-1R	5′-TATTCATAACTCCAGTGTTGTCTT-3′	R
CI21-2F	5′-TCTGTGTTTGTTTTTCTTCACGTT-3′	F
CI23-2R	5′-ATCATTCGGTTATAATGTCGTCCT-3′	R
CIT1F	5′-AAGTAGGTCTTGTAGTTTGGCTTAGCA-3′	F
CIT5R	5′-CTTCCTTCTTCTCTCCTCCCTTCTTG-3′	R

NOT1 was used for first-strand synthesis. AP1 and AP2 were used for second-strand synthesis. Primers CI8F, CI24-1F, CI25-1R, and CI22-1R were used for *Ci-gnrh1* and CI21-2F and CI23-2R were used for *Ci-gnrh2*. Primers CIT1F and CIT5R were used for the troponin control.

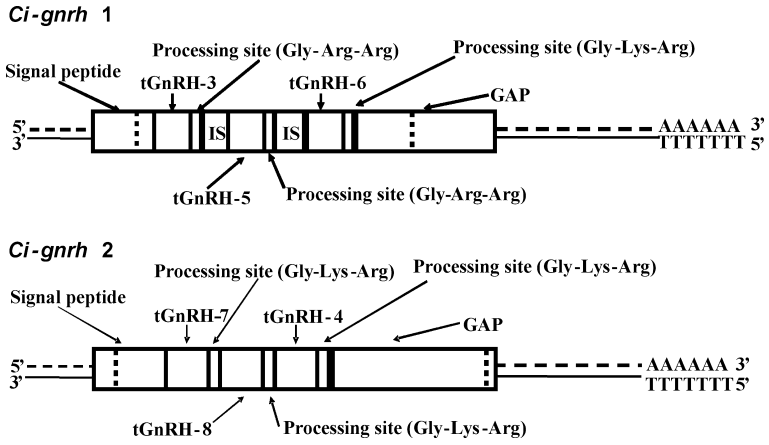

Fig. 1. Schematic diagram of the inclusive regions of *Ci-gnrh1* and *Ci-gnrh2* for PCR, RT-PCR, and in situ. *Ci-gnrh1* was designed to include the three different GnRH peptides, the intervening sequences of thirteen and eight amino acids, respectively, the three dibasic processing sites, and half of the GnRH associated peptide. *Ci-gnrh2* was designed to include the three different GnRH peptides the three dibasic processing sites and the majority of the GnRH associated peptide. Vertical doted lines indicate approximate position of primers.

Master Gradient thermal cycler using the following parameters: 48 °C for 45 min, 95 °C for 2 min followed by 35 cycles of 94 °C for 1 min, primer specific annealing temperature for 1 min, 72 °C for 1 min, finishing with a 10 min 72 °C incubation and 4 °C hold. Samples were analyzed by electrophoresis using 2% agarose gels stained with ethidium bromide, and visualized using the Molecular Imager FX (Bio-Rad, Hercules, CA).

2.5. In situ hybridization

Initial in situ hybridization was done using a digoxigenin-labeled RNA probe based on a cDNA sequence for GnRH in *C. intestinalis* Accession No. AY186743. Based on these sequences and the identified sequences of tunicate GnRHs of Adams et al. (2003), probes were designed to include both *Ci-gnrh*1 and *Ci-gnrh*2 for dual label fluorescence microscopy.

2.6. Digoxigenin-labeled RNA probe synthesis for in situ hybridization

Complementary DNAs of *C.i. Ci-gnrh1* and *Ci-gnrh2* were prepared to encompass the regions encoding for each GnRH triplet (Fig. 1) for Digoxigenin- (DIG; Roche, Indianapolis, IN) labeled riboprobe synthesis. *Ci-gnrh1* and *Ci-gnrh2* were prepared separately from reverse transcription of total RNA. Total RNA was isolated as described previously (above) using Tri-Reagent.

First-strand cDNA was constructed using the 1st Strand cDNA Synthesis Kit from Amersham–Pharmacia (Buckinghamshire, England, UK) with neural tissue total RNA as template. This first strand cDNA was then used as template for PCR with combinations of gene-specific primers designed to the GnRH triplet coding region (Table 2). These reactions were performed using a Perkin–Elmer thermal cycler under the following conditions: activation step of 94 °C for 7 min; 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min, and a 72 °C extension for 10 min followed with 4 °C hold. PCR were analyzed on 2% agarose gel stained with ethidium bromide.

All PCR products visualized on agarose gel were cloned as follows: PCR products were gel purified using the Qiaex II Gel Purification Kit and protocol from Qiagen (Valencia, CA). *Ci-gnrh1* and *Ci-gnrh2* purified PCR products were then inserted into the pGEM-T Easy vector (Promega, Madison, WI) and subsequently transformed, into JM1090 cells using the pGEM T-easy Vector System (Promega). Plasmid preparation was done with the Wizard Plus Miniprep system (Promega), following the protocol provided. Five microliters of purified plasmid for each original PCR product was then digested overnight at 37 °C with either *Sal*I or *Nco*I (2 μg BSA and 1× buffer D) (Promega) restriction enzymes producing singly digested linearized plasmids. The digestions were then analyzed by 2% agarose gel electrophoresis to confirm the presence of clones and to quantitate the DNA fragments for sequencing preparation. DNA sequencing was performed by The Huntsman Cancer Institute DNA Sequencing Facility at the University of Utah.

Digoxigenin-labeled riboprobes were synthesized using the SP6 Riboprobe Synthesis (Promega), and Digoxigenin labeled UTP as previously described (Rubin et al., 1995, 1997). This protocol was slightly modified in that the transcription reactions were allowed to continue overnight at 37 °C instead of 2 h.

2.7. In situ hybridization with fluorescence dual label

To further compare the relationship between *Ci-gnrh1* and *Ci-gnrh2* in the neural tissue of *C. intestinalis*, we examined mRNA expression of *Ci-gnrh1* and *Ci-gnrh2* within the same tissue using fluorescently labeled

riboprobes and examined by fluorescent microscopy. Riboprobes labeled with either Digoxigenin or Fluorescein (Roche) were prepared as described above. The neural tissue was cut at 14 μm in a cryostat then dehydrated and delipidated as described (Rubin et al., 1997). Signal detection used a modified protocol from (Xi et al., 2003) and described in (Root et al., 2004). Modifications included the addition of a 30 min soak in 0.3% hydrogen peroxide in methanol wash before blocking step and between signal amplification steps to eliminate endogenous peroxidase and remaining horseradish peroxidase (HRP) activity respectively, as well as the use of counterstains previously described. Visualization was performed using an Olympus BH2 microscope.

2.8. Cross-reactivity screening of in situ hybridization riboprobes

Cross-reactivity between anti-sense probes was measured using a modified Digoxigenin Northern blot protocol (Allen et al., 2000). In place of total RNA, full-length probe template cDNA used for each riboprobe synthesis was cross-linked to a nylon membrane and hybridized with the different anti-sense and sense riboprobes. Cross-reactivity was measured colorimetrically. Briefly, template cDNA was denatured for 2 min at 100 °C, spotted onto nylon membrane and UV cross-linked. Hybridization solution (50% dextran sulfate, 4× SSC, 1× Denhardt's, 1 mg/ml yeast tRNA, 10 mM DTT) were prepared and mixed with 0.5 mg/ml DIG-labeled RNA diluted in DEPC water. Probes were applied to each template cDNA and allowed to hybridize overnight at 55 °C. Following hybridization, membranes were washed twice in 2× SSC for 5 min each at RT, 1× SSC for 15 min at 55 °C, and maleate buffer (0.1 M maleaic acid, 0.3% Tween, and 0.15 M NaCl, pH 7.5) for 2 min at RT. Membranes were blocked in blocking buffer for 30 min at RT and then incubated in anti-DIG-alkaline phosphatase (AP, Roche) antibody (1:5000) for 30 min at RT. Membranes were washed twice in maleate buffer twice for 15 min each at RT, and then incubated in nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP (Roche) 1:50 diluted in 100 mM Tris–HCl, 100 mM NaCl, 50 mM Mg_2Cl_2; pH 9.5) in darkness for 1–3 h at RT. Cross-reactivity was determined by the presence of dark purple precipitate. No cross-reactivity was seen with either of the probes used in this study.

3. Results

3.1. PCR of cDNA

The amplified products obtained through PCR encoded the 5′ untranslated region through the polyadenylation tail. From these sequences, primers were chosen to produce amplicons of a length that would be used for both RT-PCR and in situ hybridization. Products for *Ci-gnrh1* and *Ci-gnrh2* were obtained, isolated and sequenced. The resulting sequences contained coding regions encoding from the signal peptide through the GAP region.

3.2. RT-PCR

Analysis of the tissue specific expression of *Ci-gnrh1* and *Ci-gnrh2* was done on heart, hepatic tissue, ovary, testes, neural tissue, total RNA, and troponin I as the control. In all tissues examined both *Ci-gnrh1* and *Ci-gnrh2* amplicons were only seen in the neural tissue (Fig. 2).

3.3. Fluorescent label in situ hybridization

Sagittal and horizontal sections (14 μm) were incubated with either anti-sense or sense digoxigenin- or fluorescein-labeled riboprobes for *Ci-gnrh1* and *Ci-*

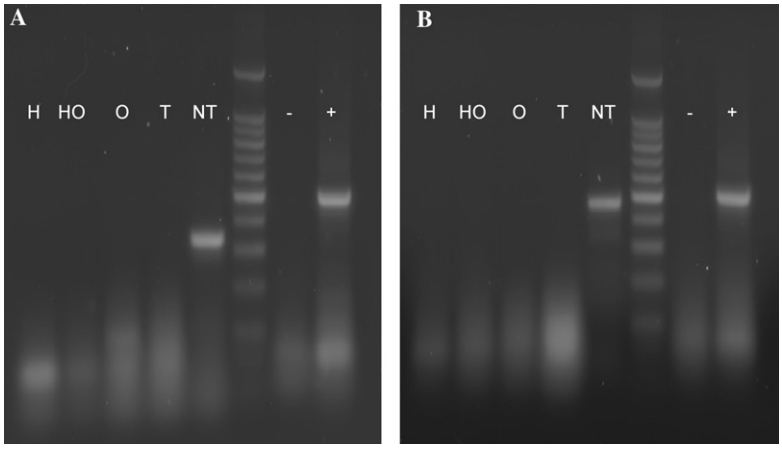

Fig. 2. RT-PCR results and controls. Expression is shown in the neural tissue of *C. intestinalis*. A is *Ci-ghrh1* and B is *Ci-gnrh2*. The − control is without template while the + control is troponin 1. The total RNA for the + control was taken from a whole animal extraction. Tissues sampled are heart (H), hepatic organ (HO), ovary (O), testis (T), and neural tissue (NT). The ladder is a Promega 100 bp ladder.

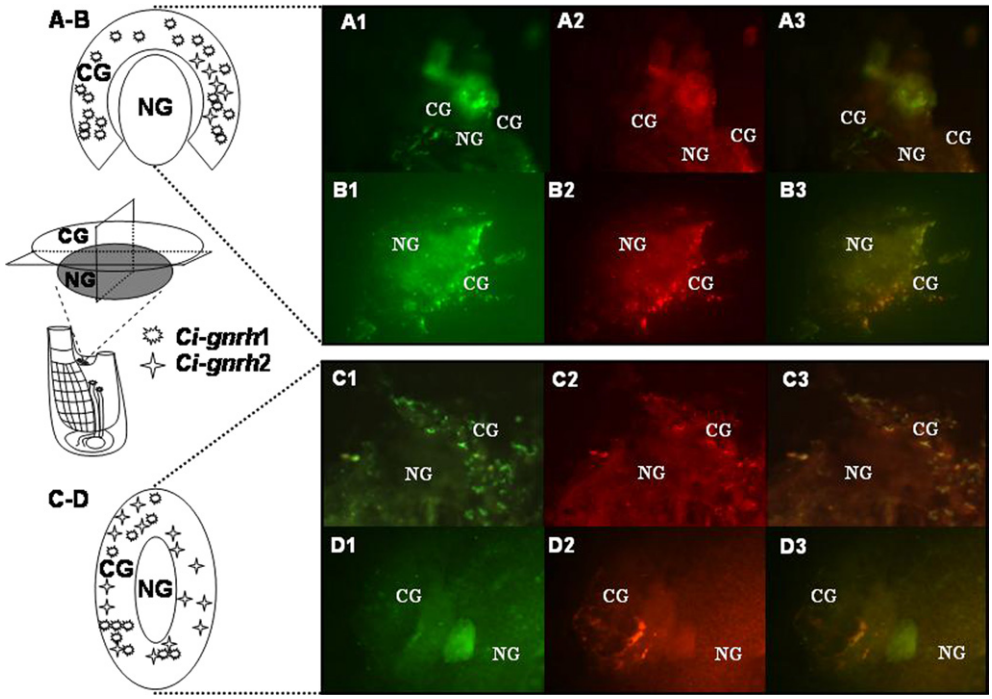

Fig. 3. Fluorescent label in situ hybridization. Digital photographs of coronal (A and B) and cross-section (C and D) tissue sections from *C. intestinalis* neural tissue, showing distinct regions expressing for digoxigenin-labeled *Ci-gnrh1* (A2, B2, C2, and D2) and fluorescein-labeled *Ci-gnrh2* (A1, B1, C1, and D1). A3, B3, C3, and D3 are overlays of *Ci-gnrh1* and *Ci-gnrh2* results to indicate similar areas of expression. CG, cerebral ganglion, and NG neural gland. (A and D 400×, B and C 200×).

gnrh2 and reaction product was allowed to develop for 3–4 h. Anti-sense probes showed strong product in only the cerebral ganglion (Fig. 3). There was no fluorescence seen in the neural gland. Tissue sections treated with respective sense probes did not show any positive results—there was little or no background. Positive expression patterns were mostly consistent in all neural tissue tested with some variance due to tissue orientation, with respect to each anti-sense probe. There was no fluorescence seen in the neural gland.

4. Discussion

The complementary DNAs encoding tunicate (*Ciona*) *Ci-gnrh1* and *Ci-gnrh2* were isolated and sequenced. These results confirm the recent cloning of the six GnRHs encoded on two genes in *C. intestinalis* (Adams et al., 2003). There is one gene *Ci-gnrh1* that encodes for tGnRH-III, -V, and -VI and the other gene *Ci-gnrh2* encodes for tGnRH-IV, -VII, and -VIII. We demonstrated in the current studies that *Ci-gnrh1* and *Ci-gnrh2* were expressed in the neural tissue as determined by in situ hybridization. More specifically using fluorescent dual label in situ hybridization, it was determined that both *Ci-gnrh1* and *Ci-gnrh2* were only expressed in the cerebral ganglion of the tissues examined. The RT-PCR data confirmed the in situ data showing *Ci-gnrh1* and *Ci-gnrh2* were only expressed in the cerebral ganglion.

These current data are supported by previous investigations that showed ir-GnRH fibers entering or leaving or both into the posterior nerve roots leading into the cerebral ganglion in *C. intestinalis* (Kelsall et al., 1990). In another ascidian, *Halocynthia roretzi*, ir-GnRH was demonstrated in the cerebral ganglion (Ohkuma et al., 2000). In ascidians, the neural complex (neural tissue) is comprised of two parts, the cerebral ganglion and neural gland (Bollner et al., 1997). In *Ciona* the cerebral ganglion is above the neural gland while in other genera it is below the neural gland (Manni et al., 1999). The findings of GnRH located in the cerebral ganglion suggest that GnRH may function as a neurotransmitter or neuromodulator since the cerebral ganglion is considered to be involved in central processing and sensory function. Whether these GnRH(s) are involved in reproduction is unknown. Although, it has been shown in various studies that a variety of treatments of synthetic tGnRH-I through -IX have all been involved in stimulating some aspect of reproduction in tunicates (Adams et al., 2003; Terakado, 2001).

The diversity of anatomical distribution patterns observed for ir-GnRH or ir-GnRH-like material in invertebrates suggests that GnRH may have multiple functions, not unlike the case for vertebrates (Gorbman and Sower, 2003). Consistent with the notion of a role of GnRH in invertebrate reproduction, a few physiological studies in a few invertebrate species have indicated that GnRH and its variants, and analogs are active in

stimulating some aspect of reproduction. In *C. intestinalis*, exogenous mammalian GnRH and chicken GnRH-I, tested in in vitro incubations of gonads, were shown to induce the release of sex steroids (Di Fiore et al., 2000), however, mammalian and chicken forms of GnRH have not been found nor the enzymes needed to synthesize sex steroids in the Ciona genome (Dehal et al., 2003). Injections of various GnRH forms, including tGnRH-I and tGnRH-II, into *C. intestinalis*, induced gamete release (Terakado, 2001). Synthetic forms of the six newly identified *C. intestinalis* GnRHs were injected into adult tunicates and all of these tunicate GnRHs induced release of gametes (Adams et al., 2003). Chang et al. (1983) demonstrated that in amphioxus, *Branchiostoma belcheri*, injection of a mammalian GnRH agonist into the body cavity resulted in an increase in estradiol and testosterone production. In mollusks, Young et al. (1997) observed that synthetic mammalian GnRH can induce an increase in egg-laying in the snail, *Helisoma trivolvis*. In later studies by these same authors, immunocytochemical studies showed that ir-GnRH cells were located in ganglion innervating the reproductive system indicating that reproduction may be regulated by GnRH in this mollusk (Young et al., 1999).

Notwithstanding, the tunicates have been the most widely studied invertebrate with regards to the identification of GnRH, its distribution and functional studies. There are likely more tissues beyond the cerebral ganglion that also produce GnRH. In previous studies, the dorsal strand plexus and gonads have been shown to contain many ir-GnRH cells (Adams et al., 2003; Georges and Dubois, 1980; Powell et al., 1996; Terakado, 2001; Tsutsui et al., 1998). In the current study, the total RNA from the whole animal produced a much brighter product independent of the amount of RNA used in the reaction (data not shown). This supports the concept that there are likely several tissues producing GnRH in *C. intestinalis*. The presence of *Ci-gnrh1* and *Ci-gnrh2* mRNA from the four cell stage through adult suggests some or all of the six encoded GnRH peptides may have roles in development outside of the reproductive axis, possibly as a neuromodulator or neurotransmitter (Adams et al., 2003). The evidence for ir-GnRH in other tissues is supported by the identification of two GnRH receptors (GnRHR) (Ci-GnRHR1 and Ci-GnRHR2) by Kusakabe et al. (2003). Both receptors were found to have similar expression patterns, both were present in the cerebral ganglion-neural gland complex, gonoduct, stomach, testis, intestine, endostyle, and branchial sac. An interesting study would be to determine in which part of the neural complex the receptors are expressed. If they were more prevalent in the neural complex this would be suggestive of a cerebral ganglion-neural gland axis.

It is well established that ascidians spawn briefly after sunrise (Lambert and Brandt, 1967; Strathmann, 1987; West and Lambert, 1976). However, the mechanism which drives gamete release through this method is not understood. It has been suggested that light might regulate the release of GnRH through retinal proteins in the cerebral ganglion (Ohkuma et al., 2000; TsuTsui and Oka, 2000). Our current findings of *Ci-gnrh1* and *Ci-gnrh2* mRNA in the cerebral ganglion in *C. intestinalis* needs to be further examined to determine the function of these GnRHs. As cited earlier, there are several physiological studies that have indicated some of the tunicate GnRHs are active in stimulating or associated with some aspect of reproduction. We have speculated that the GnRH control of reproduction may have evolved in the invertebrates for the same role as a link between the nervous system and the reproductive system (Gorbman and Sower, 2003). What will be needed are further studies to gain an understanding of the primary functions of each of the nine tunicate GnRHs and their relationship to reproduction.

Acknowledgments

This research was supported by the National Science Foundation (0090852 and 0421923 to SAS). We thank the late Dr. Aubrey Gorbman and Dr. Arthur Whiteley for their support during the initial experiments at Friday Harbour Laboratories. We also thank Dr. Larry Harris for his help in identification and collection of *C. intestinalis*. This is Scientific Contribution No. 2240 from the New Hampshire Agricultural Experiment Station.

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