The presence and distribution of gonadotropin-releasing hormone-liked factor in the central nervous system of the black tiger shrimp, *Penaeus monodon*

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Abstract

The distribution and presence of gonadotropin-releasing hormone (GnRH) in the central nervous system (CNS) of *Penaeus monodon* were examined by immunocytochemistry, high performance liquid chromatography (HPLC), and radioimmunoassay (RIA). We demonstrated the existence of octopus (oct)GnRH-liked immunoreactivity (ir-octGnRH) and lamprey (l)GnRH-III-liked immunoreactivity (ir-lGnRH-III) in cell bodies of medium-sized neurons of the anterior part (protocerebrum) of the supraesophageal ganglion (brain). In addition, only the ir-octGnRH was detected in the nerve fibers located in the brain and segmental ganglia (subesophageal, thoracic, and abdominal ganglia). Moreover, some branches of these fibers also innervated the neurons in the middle (deutrocerebrum), posterior (tritocerebrum) brain and segmental ganglia. There was no ir-lGnRH-I and ir-salmon (s)GnRH detected in the shrimp CNS. The results from HPLC and RIA showed ir-GnRH in the CNS using anti-lGnRH-III, but not with anti-mammalian (m)GnRH. The data from immunocytochemistry, HPLC and RIA suggest that ir-GnRH in shrimp may be more similar to octGnRH and lGnRH-III than the other forms. These findings support the hypothesis that GnRH-liked factor(s) may be an ancient peptide that also exists in this decapod crustacean.

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1. Introduction

GnRH is a well-known decapeptide that is a major mediator in the brain–pituitary–gonadal axis in vertebrates (Fernald and White, 1999; Morgan and Millar, 2004; Millar, 2005). Fourteen isoforms of GnRH have been reported in vertebrates and have been classified into three groups: GnRH1, 2, and 3 (Fernald and White, 1999; Tsai, 2006). Silver et al. (2004) proposed that there is the fourth group of vertebrate GnRH based on phylogenetic analysis, function, neuronal distribution, and developmental origin. In tetrapods, GnRH1 is synthesized in neurons of forebrain, transported to median eminence, and finally released into hypothalamo-hypophyseal portal circulation to stimulate the release of gonadotropins, i.e., follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary. GnRH2 is localized in the midbrain, and is speculated to serve as neurotransmitter/neuromodulator (Tsai, 2006). GnRH3 which is present in the telencephalon and the terminal nerves could be involved in sex-associated behaviors (Fernald and White, 1999). The lamprey GnRHs (lGnRH-I and -III) form the fourth group, GnRH4, which are both hypothalamic neurohormone and are derived from diencephalon/ventricular origin (Silver et al., 2004). In addition, GnRH is also detected in extra-pituitary tissues such as gonad, liver, kidney, placenta, breast, and prostate gland (González-Martínez et al., 2004).
Recently, many studies have reported the presence of GnRH in several invertebrates, which implies that this peptide could be conserved structurally as well as functionally throughout animal phyla (Young et al., 1999; Anctil, 2000; Tsai et al., 2003; Gorbman and Sower, 2003). Ten GnRH isoforms have been isolated and their primary structures determined in invertebrates, i.e., nine in tunicates and one in octopus (Powell et al., 1996; Iwakoshi et al., 2002; Adams et al., 2003; Kavanaugh et al., 2005). GnRH-like immunoreactivities have also been reported in an ascidian (Tsutsui et al., 1998), mollusks (Goldberg et al., 1993; Di Cosmo and Di Cristo, 1998; Young et al., 1999; Zhang et al., 2000; Di Cristo et al., 2002; Iwakoshi et al., 2002; Tsai et al., 2003; Iwakoshi-Ukena et al., 2004), a cnidarian (Anctil, 2000), a platyhelminthes (Anctil and Tekaya, 2005), and a coral (Twan et al., 2006). Although the structures have not yet been identified, the existence of the ir-GnRH in a variety of invertebrates suggests the ancestral GnRH has predated the emergence of vertebrate, and that GnRH is an ancient peptide that exists throughout vertebrate and invertebrate phyla (Rastogi et al., 2002; Gorbman and Sower, 2003; Tsai, 2006). GnRH has been reported to be functionally conserved as a reproduction-regulating factor in a few invertebrate species (Fang et al., 1991; Young et al., 1999; Zhang et al., 2000; Adams et al., 2003; Gorbman et al., 2003; Iwakoshi-Ukena et al., 2004; Twan et al., 2006). In this report, we demonstrated ir-GnRH present in the CNS of the black tiger shrimp, Penaeus monodon, which may suggest the involvement of GnRH in the shrimp reproduction.

2. Materials and methods

2.1. Animals

Sexually mature female shrimp, with average weight of 180–250 g were caught from the Gulf of Thailand, and maintained at the Department of Aquatic Science, Faculty of Science, Burapha University, Chonburi, Thailand. The shrimp were maintained in a cement tank filled with seawater, at a temperature of about 25–28°C, salinity at 30 ppt, with continuous aeration. They were fed with mince squid, and kept under a normal day/night cycle (12/12 h). Approximately 70% of the seawater was replaced once a day. The shrimp were acclimatized for at least 7 days before being sacrificed. For chromatographic and radioimmunoanalytical studies, 30 whole CNS (supraesophageal and segmental ganglia) were dissected, and immediately frozen in liquid nitrogen and stored at −80°C until use.

2.2. Antibodies and GnRH peptides

For immunocytochemistry, four available antibodies against GnRHs were used. The first was antibody against octGnRH (anti-octGnRH, Lot 9779, generously provided by Dr. Pei-San Tsai). This antibody was selected since octopus is considered to be more related to shrimp than crabs as both are protostomes. The other three antibodies were generated from one basal vertebrate (lamprey) and one basal teleost (salmon) which included anti-sGnRH, Lot 1667 (a kind gift of Dr. Judy King), anti-mGnRH-I, Lot 1467, and anti-IgG-RH-III, Lot 3952 (produced in the laboratory of Dr. Stacia A. Sower, Sower et al., 1993). The lamprey as a basal vertebrate may have retained ancestral characteristics found in invertebrate GnRHs. The sGnRH is considered as GnRH3, whereas the IgG-RHs are classified as GnRH4. Antibody against mGnRH (GnRH-I, Lot R1245) was also used for determining the existence of a mGnRH-like peptide in the RIA. The RIA using anti-mGnRH showed no immunoreactivity (see Section 3), thus this antibody was excluded from further use in immunocytochemistry. For peptides used in preabsorption study, octGnRH peptide (a gift from Dr. Hiroyuki Minakata and Dr. Pei-San Tsai), IgG-RH-I, and IgG-RH-III peptides (from the laboratory of Dr. Stacia A. Sower, purchased from American Peptide at 95% purity) were used.

2.3. Immunocytochemistry

The eyestalk, supraesophageal ganglion, and segmental ganglia were removed from non-gravid female shrimps. They were fixed in Bouin’s fixative overnight, dehydrated, embedded in paraffin blocks, and then sectioned at 7 μm thick. The sections were deparaffinized with xylene, rehydrated through a graded series of ethanol (100–70%). The sections were immersed in 1% H2O2 in 70% ethanol for 15 min to eliminate endogenous peroxidase, then covered with 0.1% glycine and 4% BSA in 0.1 M phosphate buffer saline (PBS), pH 7.4, for 15 min for blocking free-alkylhyde and non-specific binding, respectively. The sections were then incubated with the primary antiserum including anti-octGnRH (1:500), anti-IgG-RH-I (1:1000), and anti-IgG-RH-III (1:4000), at 4°C for overnight. Negative controls were performed by incubating the sections in PBS or in primary antiserum which were preabsorbed with GnRH peptides (50 μg/0.1 ml antiserum at working dilution). Thereafter, sections were rinsed in several baths of PBS containing 0.1% Tween-20, and subsequently incubated with peroxidase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) diluted at 1:1500 for 1 h. The presence of ir-GnRH in the tissues was enhanced and visualized by DAB enhanced liquid substrate system (Sigma). The sections were then dehydrated, cleared, mounted, and observed under a light microscope. The experiments were repeated for at least three times for each tissue. In addition, some sections were processed by conventional method, and then stained with hematoxylin (H) and eosin (E) dyes for histological characterization of the tissues being studied.

In order to compare the staining intensity of ir-GnRH in experimental and controlled sections which were probed with preabsorbed anti-GnRHs, densitometric analysis of stained neurons was performed using ImageJ software. A box of 50 × 50 pixels was generated and placed over the positively-stained areas. On the same section, the areas which showed negative staining were also measured and considered as background staining. These background values were then used to subtract from the values measured from the positively-stained areas. At least 10 areas from both positive and negative immunoreactive areas were randomly measured in one section, and three sections from each experiment were analyzed.

2.4. GnRH peptide extraction and HPLC analysis

The shrimp CNS were extracted according to the methods described by Fahien and Sower (1990) at the Faculty of Science, Mahidol University, Thailand. Briefly, the CNS parts were weighed, and then homogenized at 4°C with a polytron in 2.0 M ice-cold acetic acid. The homogenate was centrifuged at 10,000 g for 45 min. The supernatant was subsequently dried in Speed Vac Concentrator, and then kept at −80°C. Further processing of the extracts was performed at the Department of Biochemistry and Molecular Biology, University of New Hampshire, USA. The extract was resuspended with Milli-Q water, and purified by a Sep-Pak C18 cartridge column pretreated with 100% methanol. The peptide was eluted from the column with 70% acetonitrile. The elute was then dried on Speed Vac Concentrator for overnight. The filtrate was subsequently injected into a 20 μl the injection loop of a Perkin-Elmer HPLC system filled with a Pecosphere 3CR C18 (0.45 mm × 8.3 cm) reverse-phase column. The isocratic mobile phase consisted of 7.4 g ammonium acetate and 3.04 g citric acid in 1000 ml of 19% acetonitrile with flow rate of 2 ml/min. The fractions were collected every 18 s.
Fig. 1. (A) A photo of a dissected shrimp and a corresponding diagram illustrating the organization of the shrimp CNS which is comprised of the supraesophageal ganglion (SupG) and segmental ganglia (subesophageal ganglion, SubG; thoracic ganglia, TG; and abdominal ganglia, AG). The segmental ganglia are connected by a ventral nerve cord which runs along the ventral axis of the shrimp body. (B) A diagram showing the locations of various neuronal clusters as numbered in the supraesophageal ganglion (modified from Sandeman et al., 1992). (C–E) Sagittal sections of the supraesophageal ganglion stained with H&E, demonstrating neuropils and neuronal cell clusters (asterisks) in protocerebrum (C), deutocerebrum (D), and tritocerebrum (E). (F) Three types of neurons: large-sized neurons (asterisks), medium-sized neurons (large arrows), and small-sized neurons (small arrows) are revealed in cluster 13. PNp, protocerebrum neuropil; DNp, deutocerebral neuropil; TNp, tritocerebral neuropil.
Mammalian GnRH, chicken (c)GnRH-I and II, octGnRH, sGnRH, and lGnRH-I and III standards were chromatographed in parallel in the same HPLC system.

2.5. Radioimmunoassay

The eluted HPLC fractions were dried on Speed Vac Concentrator and then resuspended in phosphate gelatin buffer before measuring the concentrations of GnRH-like factor using RIA as previously described by Fahien and Sower (1990). The HPLC fractions were assayed in duplicate. The synthetic mGnRH or lGnRH-I was used as the radio-iodinated tracer and standard. The anti-mGnRH and anti-lGnRH-II were used at dilutions of 1:30,000 for mGnRH RIA and 1:16,000 for lGnRH-III RIA, respectively. The antibody binding ranged between 26–32% for anti-mGnRH of 125I-mGnRH and 50–59% for anti-lGnRH-II of 125I-lGnRH. Anti-mGnRH has a specificity with cross-reactivities of 100%, 65%, 19.5%, 4.16%, and <0.0001% for mGnRH, cGnRH-I, sGnRH, cGnRH-II, and lGnRH-I, respectively (Calvin et al., 1993; Sower et al., 1995). The anti-lGnRH-II has cross-reactivities of 100% with lGnRH-I and III, <0.01% with mGnRH, cGnRH-I, cGnRH-II, and sGnRH (Robinson et al., 2000), and 0.1% with octGnRH (unpublished data). Standard curves were constructed by assaying the standard GnRHs that ranged from 0 to 2500 pg for lGnRH-III assay and 0 to 1250 pg for mGnRH assay. Lower limit of detection in each assay was 9.8 pg/0.1 ml. A RIA for anti-octGnRH has not yet been developed.

3. Results

3.1. Immunocytochemistry

The shrimp CNS comprised of supraesophageal ganglion and segmental ganglia, and the latter were divided into subesophageal, thoracic, and abdominal ganglia (Fig. 1A). The shrimp supraesophageal ganglion (comprising of protocerebrum, deutocebrum, and tritocerebrum) is organized as the neuropils and clusters of neuronal cells (Fig. 1B–E). Neuronal cells in each cluster were classified into three types based on their sizes: small-, medium-, and large-sized neurons which ranged about 7–15, 20–40, and 50–150 μm, respectively (Fig. 1F). Four antisera as mentioned in Section 2 were applied as probes to determine the presence of ir-GnRH in this part of the shrimp CNS. Anti-octGnRH exhibited the most intense staining. There was no ir-octGnRH observed in neuronal cell bodies, fibers, or neuropils of the eyestalk (Fig. 2A and B). However, ir-octGnRH was detected in cell bodies of medium-sized neurons located in the anterior part of the brain, the protocerebrum, and in fibers distributed in the protocerebrum (Fig. 3A and B). There was no ir-octGnRH observed in cell bodies of neurons located in the deutocebrum, tritocerebrum and segmental ganglia. In contrast, intense ir-octGnRH was detected in the fibers surrounding the neuropils of deutocebrum and tritocerebrum, and also in the fibers that innervated neuronal cell bodies in these two parts of brain (Fig. 3C–F). The fibers innervated neuronal cell bodies in segmental ganglia also exhibited intense ir-octGnRH (Fig. 4). There was no ir-octGnRH observed in the sections incubated with preabsorbed anti-octGnRH (Fig. 3G).

The ir-lGnRH-III was found only in the cell bodies of medium-sized neurons located in protocerebrum (Fig. 5A). However, the immunostaining was less intense, and there were fewer immunoreactive neuronal cell bodies when compared to the ir-octGnRH positive neurons. In addition, neither neuronal cell bodies nor fibers in eyestalk and segmental ganglia exhibited ir-lGnRH-III. Interestingly, ir-octGnRH and ir-lGnRH-III were detected in neuronal cell bodies of neuronal cluster number 6 only, while the closely associated neuronal clusters numbered 7 and 8 were not stained. There was no ir-lGnRH-I or ir-sGnRH were detected in any other parts of the shrimp CNS. The presence of each ir-GnRH isoform in each section of the shrimp CNS is summarized in Table 1. There was no ir-lGnRH-III observed in the sections incubated with preabsorbed anti-lGnRH-III (Fig. 5B).

The specificities and intensities of anti-octGnRH and anti-lGnRH-III staining were compared, and the results are summarized in Table 2. Anti-octGnRH exhibited intense staining in the shrimp CNS with the intensity of 76.00 ± 1.73 U. After preabsorption of this antibody with octGnRH peptide, the intensity was reduced to 3.70 ± 0.82 U, suggesting that this antibody was highly specific to octGnRH. However, approximately 50% reduction in the intensity (39.10 ± 4.06 U) was observed after preabsorption with lGnRH-III (Table 2).

Fig. 2. Immunocytochemical localization of ir-octGnRH in the shrimp eyestalk. No ir-octGnRH is detected in neurons and neuropils of the optic lobe (A and B). MT, medulla terminalis.
Fig. 3. Immunocytochemical localization of ir-octGnRH in the shrimp supraesophageal ganglion. Intense immunoreactivity is detected in some medium-sized neurons located in protocerebrum (A and B, large arrows) and in the neuronal fibers (arrow head). Other medium-sized neurons (asterisk) and all of the small-sized neurons (small arrows) in this part of the ganglion show no immunoreactivity. The ir-octGnRH is also detected in the fibers (C–F, arrows) that innervated neurons (asterisks), as well as the fibers surrounded the neuropils of deutocerebrum and tritocerebrums (E, inset, arrow head). (G) The section incubated with preabsorbed anti-octGnRH shows only background staining. DNp, deutocerebral neuropil; PNp, protocerebral neuropil; TNp, tritocerebral neuropil.
preabsorbing the antibody with lGnRH-III peptide. Without preabsorption, the anti-lGnRH-III exhibited moderate staining in the shrimp CNS with the intensity of 55.60 ± 1.18 U. After preabsorption with lGnRH-I and lGnRH-III peptides, this antibody was totally blocked resulting in background staining only (3.17 ± 0.60 and 1.47 ± 0.38 U, respectively). In contrast, when the anti-lGnRH-III was preabsorbed with octGnRH, there were

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Fig. 4. Immunocytochemical localization of ir-octGnRH in subesophageal (A and B), thoracic (C and D), and abdominal ganglia (E and F). The ir-octGnRH is observed in the subesophageal neuropil (SuNp), thoracic ganglion neuropil (TgNp), abdominal ganglion neuropil (AgNp), and in fibers (arrows) innervating the neurons (asterisks) located in the segmental ganglia. D, dorsal; R, rostral.
very low cross-reactivity with octGnRH (51.80 ± 1.48 U), and the antibody still exhibited the usual staining.

3.2. HPLC and RIA

In the HPLC and RIA analyses from CNS extract using the anti-lGnRH-III, there was only one major peak of ir-GnRH (Fig. 6). The elution time of this fraction was compared to the elution times of seven different synthetic GnRH standards run in parallel on the same HPLC system. It was shown that the elution position of the ir-GnRH peak was about 18 and 54 s earlier than those of lGnRH-III and octGnRH standards, respectively. This suggested that the shrimp CNS contained at least one GnRH-liked factor that may be closely related to lGnRH-III.

4. Discussion

In this study, we demonstrated the existence of GnRH-liked factor(s) in a penaeid shrimp CNS by immunocytochemistry using four different types of GnRH antibodies. Ir-octGnRH and ir-lGnRH-III were detected in the neuronal cell bodies located in protocerebrum. Considering that anti-lGnRH-III and anti-octGnRH showed about 0.1% cross-reactivity (unpublished data), we propose that there may be at least two isoforms of GnRH in the shrimp CNS. These findings were supported by the results obtained from the RIA assay of individual HPLC fractions which revealed the existence of at least one GnRH-liked factor that is closely related to lGnRH-III in the shrimp CNS. Unfortunately, the RIA system for ir-octGnRH has not yet been developed, thus we could not confirm
mandibular organ-inhibiting hormone (MOIH) are synthesized (Huberman, 2000). Functionally, MIH inhibits secretion of a molting hormone, ecdysone produced by the Y-organ. GIH is shown to inhibit ovarian development and vitellogenin synthesis, whereas MOIH acts on mandibular organ by inhibiting the secretion of methyl-farnesoate (MF), which is believed to stimulate vitellogenesis and ecdysteroid synthesis in Y-organ (Okumura and Aida, 2001). Since we have shown that there is no apparent ir-GnRH in the eyestalk, we postulate that GnRH may be confined to the distal part of the shrimp nervous system and subjected to the regulation by the inhibiting factors present in the eyestalk. However, the identification of the primary structures of shrimp GnRH and the possible interrelationships of GnRH and the eyestalk inhibiting factors will need to be studied before any firm conclusion can be reached.

In vertebrates, GnRH neurons are neuroendocrine cells that release their contents to affect a large number of target cells (Goldberg et al., 1993). The significance of GnRH in regulating reproduction has been well defined in all vertebrates and suggested for some invertebrates (Fang et al., 1991; Young et al., 1999; Di Fiore et al., 2000; Zhang et al., 2000; Adams et al., 2003; Gorbman and Sower, 2003; Gorbman et al., 2003; Iwakoshi-Ukena et al., 2004; Twan et al., 2006). Although the protocerebrum contains many neuronal cell clusters (Sandeman et al., 1992), our present study demonstrated ir-octGnRH and ir-lGnRH-III only in cell bodies of medium-sized neurons of neuronal cluster 6 that is located in the most anterior part of the shrimp brain. In decapod crustaceans, these neurons are also considered to be "neurosecretory cells" (Bell and Lightner, 1988). Similarly in a mollusk, Halisoma trivolvis, the neuronal cells involved in controlling reproduction were shown to be located in the anterior part of the nervous system especially in the cerebral ganglia, and that this ganglion was shown to be the site for ir-GnRH neurons (Goldberg et al., 1993). By contrast, it is well known that deutocerebrum and tritocerebrum are mainly involved in olfaction and mechanosensory functions, respectively (Sandeman et al., 1992). In Aplysia californica, prior to spawning, stimulatory signals originating from the head ganglia are transmitted to the bag cells (neurons) in the abdominal ganglion to trigger the release of egg-laying hormone (ELH), thus initiating egg-laying behavior and spawning (Wayne, 2001). In shrimp, these functions are known to be controlled in part by gonad-stimulating hormone (GSH) secreted from brain and/or thoracic ganglia (Huberman, 2000). The GSH was suggested to be regulated by an increased level of serotonin (Meeratana et al., 2006; Wongprasert et al., 2006). It is also possible that GnRH may be involved in this pathway. However, the interrelationships of GnRH and GSH will need further study.

Our immunocytochemical results showed that the ir-GnRH(s) were detected exclusively in the cell bodies of neurons located in the protocerebrum, while neuronal fibers with ir-GnRH were observed to innervate neurons
in the deuto cerebrum, tritocerebrum, and segmental ganglia. This suggests that GnRH produced in protocerebrum may be passed caudally to exercise its reproductive control on the more distally located neurons that may be involved in the production and release of final effector neurohormone such as GSH. We have started initial investigations on the potential role of GnRH-like factor in mediating reproductive function in this species of shrimp. Results from these ongoing studies and future structural-functional studies will provide much needed information on GnRH in an invertebrate. In summary, we have detected ir-GnRH in the CNS of shrimp, whether these GnRH(s) are directly involved in reproduction has yet to be determined.

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