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; Di Fiore et al., 2000; 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 minced squid and kept under a normal day/light 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 radioimmunological 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 immunocytochemitry, 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 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-lGnRH-I, Lot 1467, and anti-lGnRH-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 ancentral characteristics found in invertebrate GnRHs. The sGnRH is considered as GnRH3, whereas the lGnRHs are classified as GnRH4. Antibody against mGnRH (GnRH1, Lot R1245) was also used for determining the existence of a mGnRH-liked 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), lGnRH-I, and lGnRH-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% H_2O_2 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-aldehyde and non-specific binding, respectively. The sections were then incubated with the primary antisera including anti-octGnRH (1:500), anti-sGnRH (1:500), anti-lGnRH-I (1:1000), and anti-lGnRH-III (1:4000), at 4 °C for overnight. Negative controls were performed by incubating the sections in PBS or in primary antisera which were preabsorbed with GnRH peptides (50 μg/0.1 ml antisera 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% acetronitrile. The elute was then dried on Speed Vac Concentrator followed by resuspension in Milli-Q water. Insoluble material was filtered using an ARCO LC 13 (0.45 μm). The filtrate was subsequently injected into a 20 μl the injection loop of a Perkin–Elmer HPLC system filled with a Percosphere 3CR C18 (0.46×8.3 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% acetronitrile with flow rate of 2 ml/min. The fractions were collected every 18 s.

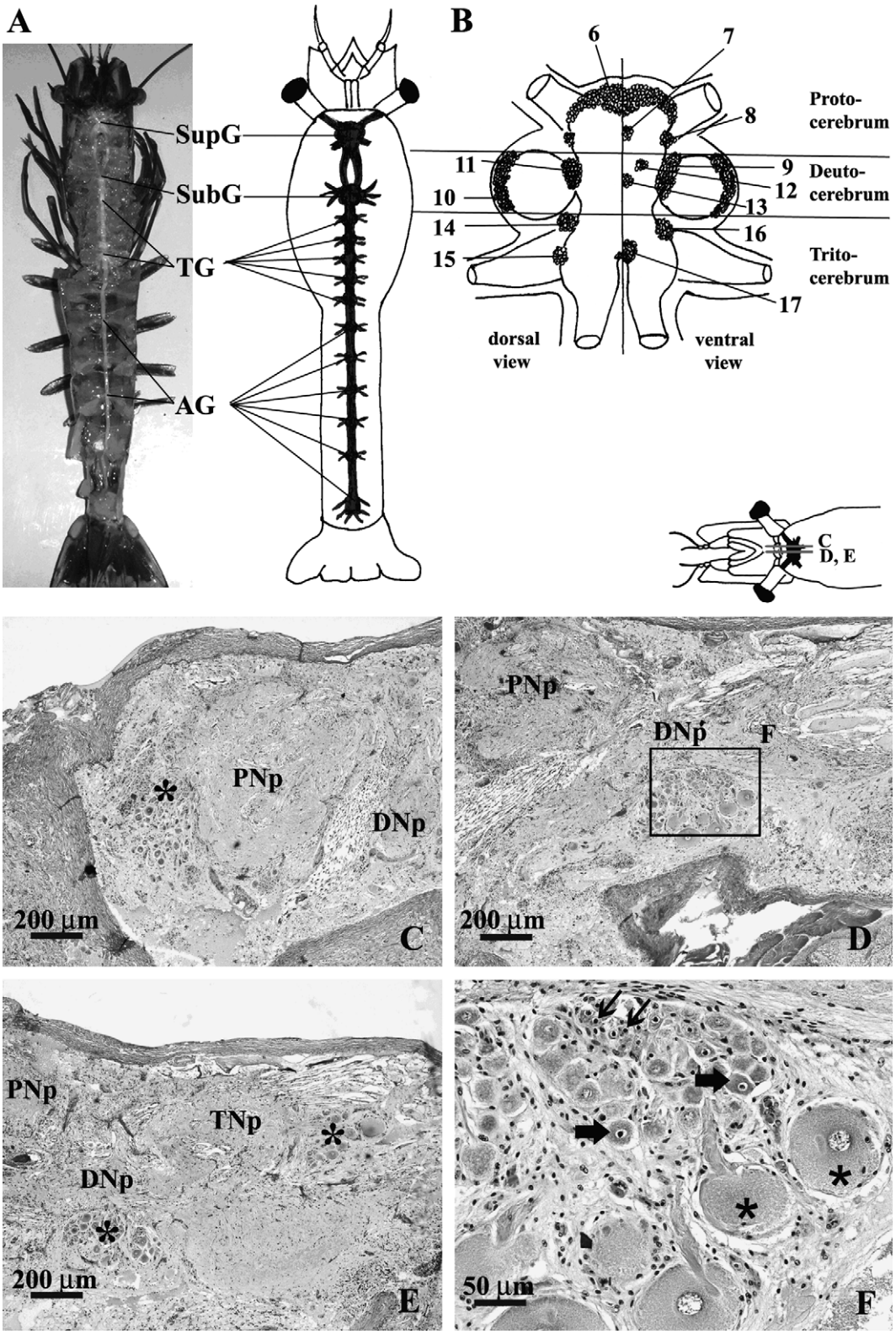

Fig. 1. (A) A photo of a dissected shrimp and a corresponding diagram illustrating the organization of the shrimp CNS which is comprised of the supraesophageal ganglion (SupG) and segmental ganglia (subesophageal ganglion, SubG; thoracic ganglia, TG; and abdominal ganglia, AG). The segmental ganglia 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-liked 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-III 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 ^{125}I-mGnRH and 50–59% for anti-lGnRH-III of ^{125}I-lGnRH. Anti-mGnRH has a specificity with cross-reactivities of 100%, 65%, 19.5%, 4.16%, and <0.00001% for mGnRH, cGnRH-I, sGnRH, cGnRH-II, and lGnRH-I, respectively (Calvin et al., 1993; Sower et al., 1995). The anti-lGnRH-III 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, deutocerebrum, 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 deutocerebrum, tritocerebrum and segmental ganglia. In contrast, intense ir-octGnRH was detected in the fibers surrounding the neuropils of deutocerebrum 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

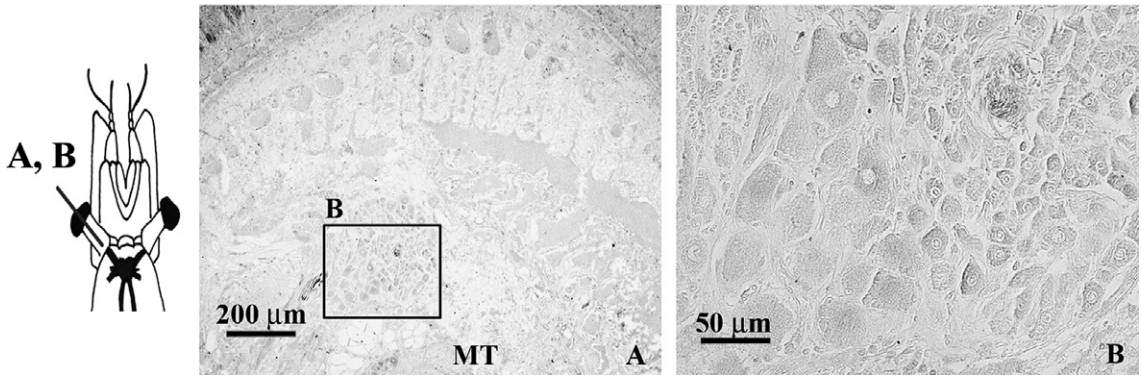

Fig. 2. Immunocytochemical localization of ir-octGnRH in the shrimp eyestalk. No ir-octGnRH is detected in neurons and neuropils of the optic lobe (A and B). MT, medulla terminalis.

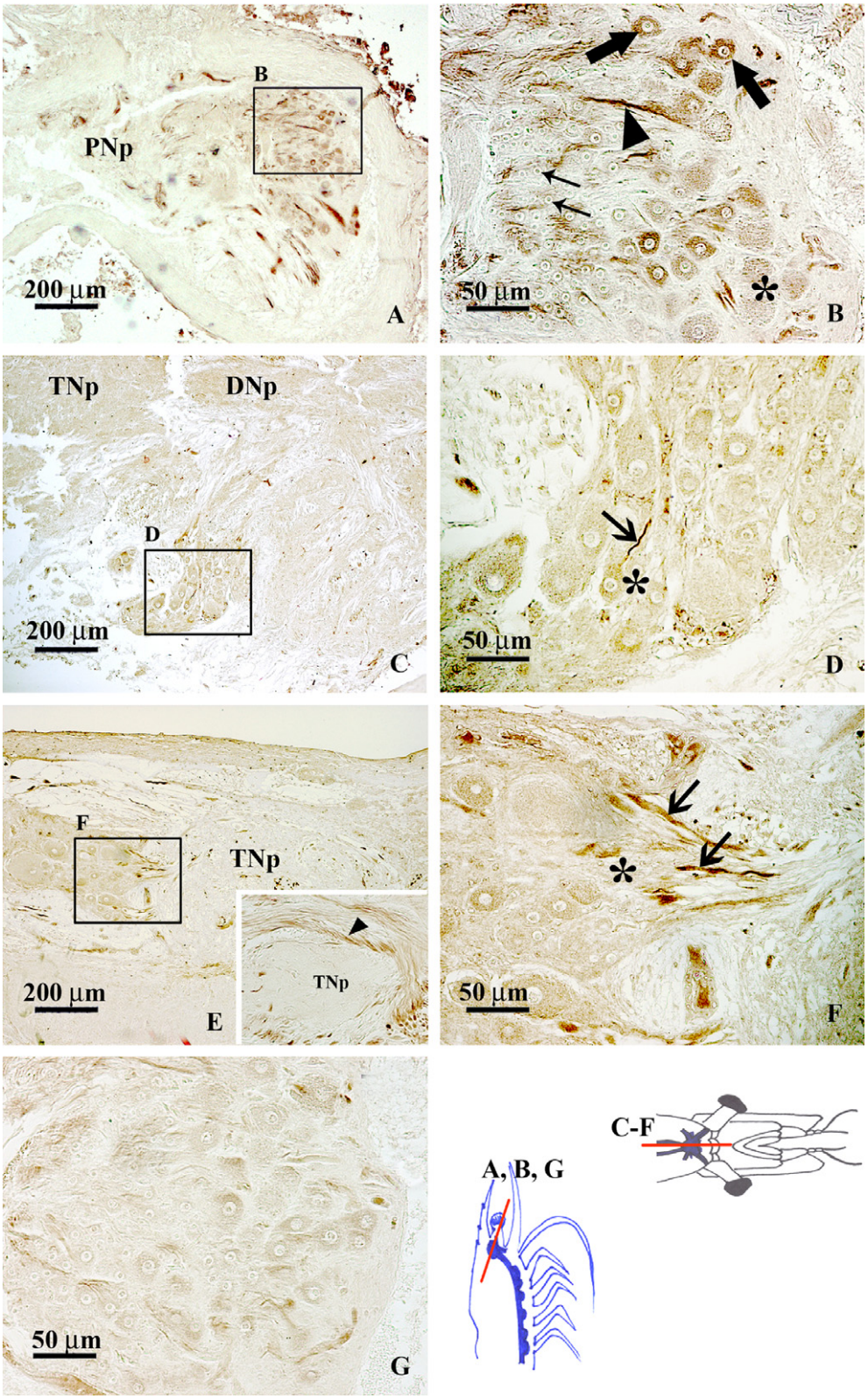

Fig. 3. Immunocytochemical localization of ir-octGnRH in the shrimp supraesophageal ganglion. Intense immunoreactivity is detected in some medium-sized neurons located in protocerebrum (A and B, large arrows) and in the neuronal fibers (arrow head). Other medium-sized neurons (asterisk) and all of the 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.

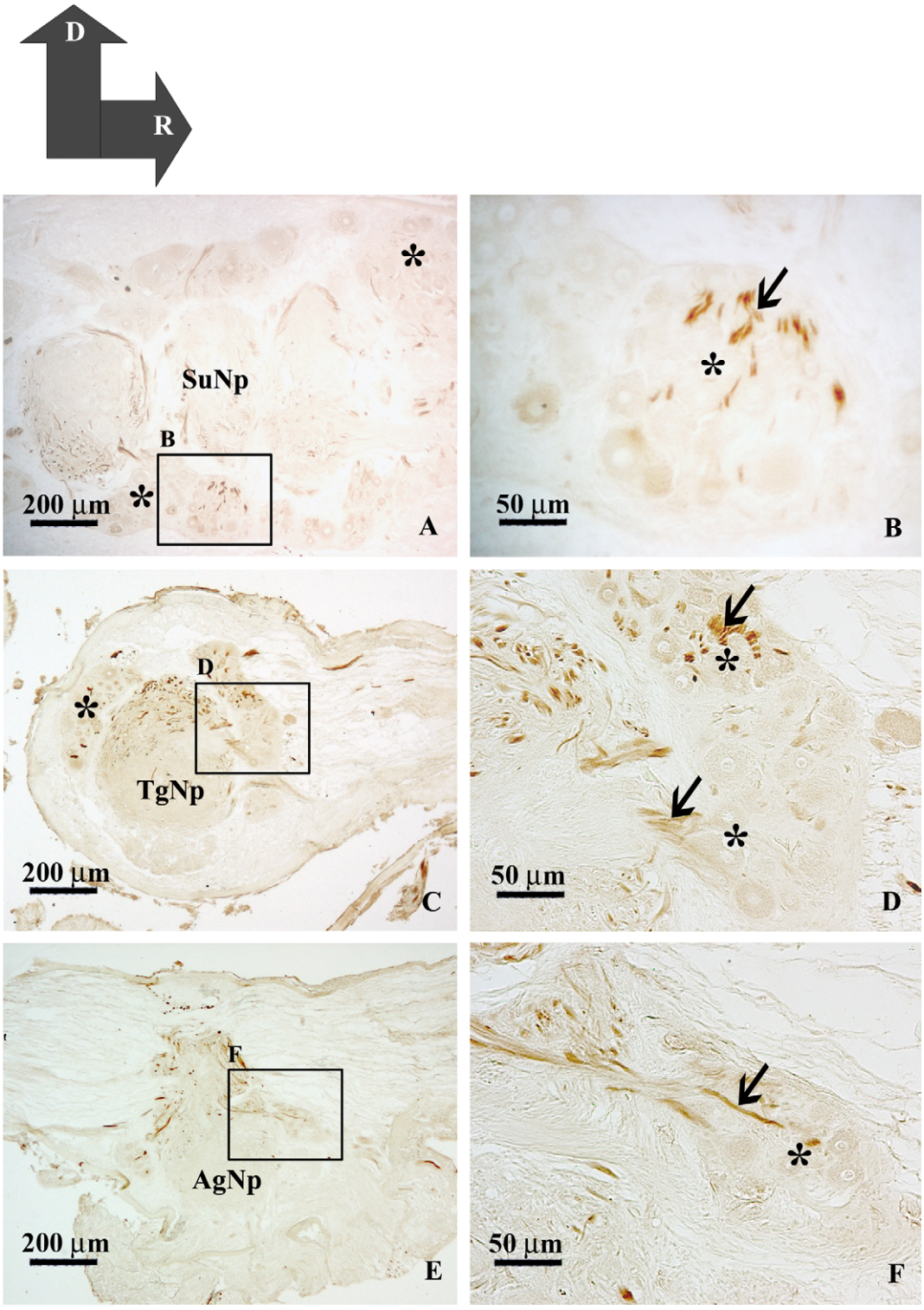

Fig. 4. Immunocytochemical localization of ir-octGnRH in subesophageal (A and B), thoracic (C and D), and abdominal ganglia (E and F). The ir-octGnRH is observed in the subesophageal neuropil (SuNp), thoracic ganglion neuropil (TgNp), abdominal ganglion neuropil (AgNp), and in fibers (arrows) innervating the neurons (asterisks) located in the segmental ganglia. D, dorsal; R, rostal.

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

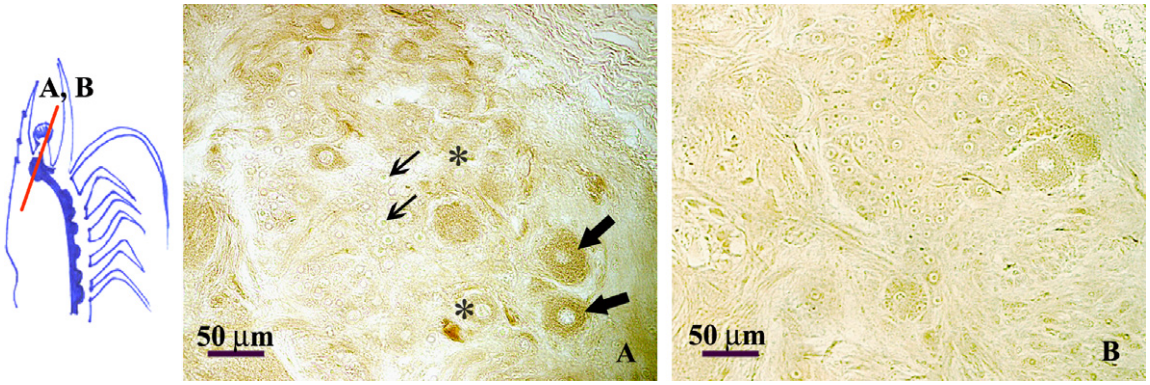

Fig. 5. Immunocytochemical localization of ir-lGnRH-III in the shrimp supraesophageal ganglion. (A) Immunoreactivity is seen in some medium-sized neurons of protocerebrum (large arrows). Other medium-sized neurons (asterisks) and all small-sized neurons (small arrows) show no immunoreactivity. (B) The section incubated with preabsorbed anti-lGnRH-III shows only background staining.

Table 1
Distribution of four GnRH isoforms in the black tiger shrimp CNS as determined by relative intensities of immunostaining with antibodies against octGnRH, lGnRH-I, lGnRH-III, and sGnRH

Antisera against	Part of CNS									
	Eyestalk		Supraesophageal ganglion						Segmental ganglia	
			Protocerebrum		Deutocerebrum		Tritocerebrum			
	Soma	Fibers	Soma	Fibers	Soma	Fibers	Soma	Fibers	Soma	Fibers
octGnRH	–	–	+++	+++	–	+++	–	+++	–	+++
lGnRH-III	–	–	++	–	–	–	–	–	–	–
lGnRH-I	–	–	–	–	–	–	–	–	–	–
sGnRH	–	–	–	–	–	–	–	–	–	–

Note. +++, intense immunostaining; ++, moderate immunostaining; +, faint immunostaining; –, no immunostaining.

Table 2
The staining intensities and specificities of antibodies before and after preabsorption with GnRH peptides exhibiting ir-GnRH in the black tiger shrimp supraesophageal ganglion

Antibodies	Lot No.	Without preabsorption	Preabsorbtion with		
			OctGnRH	lGnRH-I	lGnRH-III
αoct-GnRH	9779	76.00 ± 1.73	3.70 ± 0.82	74.93 ± 1.06	39.10 ± 4.06
αlGnRH-III	3952	55.60 ± 1.18	51.80 ± 1.48	3.17 ± 0.60	1.47 ± 0.38

The numbers represent the units of staining intensities obtained by subtracting values of background staining from positive one.

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

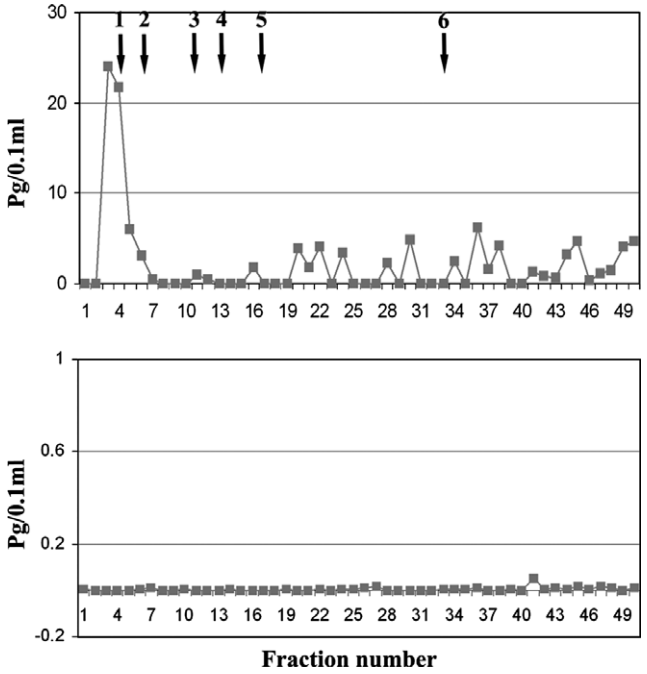

Fig. 6. RIA of HPLC fractions using anti-lGnRH-III (top) and anti-mGnRH (bottom). Arrows indicate elution times of standard GnRHs in the following order: 1, lGnRH-III; 2, octGnRH; 3, mGnRH/cGnRH-I; 4, lGnRH-I; 5, cGnRH-II; 6, sGnRH. The elution position of the shrimp ir-GnRH is about 18 and 54 s earlier than the positions of lGnRH-III and octGnRH standards, respectively.

the presence of octGnRH-liked factor in the shrimp CNS by this method.

Immunocytochemistry showed stronger intensity of ir-octGnRH in both neuronal cell bodies and fibers than that exhibited by ir-lGnRH-III. This implies the presence of more octGnRH-liked factor in the shrimp CNS than that of lGnRH-III-liked factor. In contrast to the presence of ir-octGnRH and ir-lGnRH-III, there was no ir-lGnRH-I or ir-sGnRH in any part of the shrimp CNS as determined by immunocytochemistry. This was consistent with the results from the HPLC and RIA studies, which did not show any ir-peaks corresponding to the peaks of the standards of lGnRH-I or sGnRH. The specificity of both ir-octGnRH and ir-lGnRH-III in the shrimp CNS was shown by preabsorption of the antisera with corresponding synthetic octGnRH and lGnRH-III peptides. In both cases, only a background level of the immunoreactivity was detected, thus confirming that these antibodies were highly specific to their corresponding GnRH-liked factors. Thus, it is suggested that there are at least two GnRH-liked peptides present in the shrimp CNS.

Interestingly, no ir-GnRH was detected in either the neuronal cell bodies or fibers of the shrimp eyestalk, especially in the optic lobe that is connected to the protocerebrum, and is considered to play an important role in the regulation of molting and reproduction. The protocerebrum is the site where a number of reproduction-regulating neurohormones including molt-inhibiting hormone (MIH), gonad-inhibiting hormone (GIH), and 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 deutocerebrum, 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-liked 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.

Acknowledgments

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ORIGINAL PAPER

The identification and distribution of gonadotropin-releasing hormone-like peptides in the central nervous system and ovary of the giant freshwater prawn, *Macrobrachium rosenbergii*

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Abstract In the present study, we demonstrated the existence of GnRH-like peptides in the central nervous system (CNS) and ovary of the giant freshwater prawn, *Macrobrachium rosenbergii* using immunocytochemistry. The immunoreactivity (ir) of lamprey (l) GnRH-III was detected in the soma of medium-sized neurons located in neuronal cluster number 11 in the middle part of supraesophageal ganglion (deutocerebrum), whereas ir-octopus (oct) GnRH was observed in the soma of both medium-sized and large-sized neurons in thoracic ganglia, as well as in the fibers innervating the other medium-sized and large-sized neuronal cell bodies in the thoracic ganglia. In addition, ir-lGnRH-I was observed in the cytoplasm of late previtellogenic oocyte and early vitellogenic oocyte. These data suggest that *M. rosenbergii* contain at least three isoforms of GnRH: two GnRH isoforms closely related to lGnRH-III and octGnRH in the CNS, whereas another isoform, closely related to lGnRH-I, was localized in the ovary. This finding provides supporting data that ir-GnRH-like peptide(s) may exist in this decapod crustacean.

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Introduction

In vertebrates, gonadotropin-releasing hormone (GnRH), a decapeptide, plays a critical role in regulating reproduction (Gorbman and Sower 2003; Morgan and Millar 2004; Tsai 2006). The major reproductive-regulating pathway of GnRH is known as the hypothalamo-pituitary-gonadal axis. GnRH is synthesized in the hypothalamic neurosecretory cells and then transported to the anterior pituitary gland via the hypophyseal portal vessels (in tetrapods) or direct innervation (in teleosts) to regulate the secretion of gonadotropins, i.e., follicle stimulating hormone (FSH) and luteinizing hormone (LH). The gonadotropins are then released into blood stream and act on the gonads to stimulate gametogenesis and steroidogenesis (Fernald and White 1999). To date, the primary structures of 14 and 11 GnRHs have been identified in vertebrates and invertebrates, respectively (Gorbman and Sower 2003; Tsai 2006; Kah et al. 2007; Zhang et al. 2007). Among invertebrate GnRHs, nine isoforms identified in the tunicates are decapeptides, whereas two isoforms discovered in the octopus and aplysia are dodecapeptides (Powell et al. 1996; Iwakoshi et al. 2002; Adams et al. 2003; Kavanaugh et al. 2005; Zhang et al. 2007).

Within the last decade, GnRH or immunoreactive GnRH-like peptides have been shown in a number of invertebrate species, which indicates that this peptide or variant exists throughout vertebrate and invertebrate phyla (Gorbman and Sower 2003; Tsai 2006; Kah et al. 2007). In mollusks, the existence of immunoreactivity (ir)-GnRH

was reported in gastropods, *Halisoma trivolvis, Lymnaea stagnalis*, and *Aplysia californica* (Goldberg et al. 1993; Pazos and Mathieu 1999; Young et al. 1999; Zhang et al. 2000, 2007; Tsai et al. 2003), and a cephalopod, *Octopus vulgaris* (Di Cosmo and Di Cristo 1998; Di Cristo et al. 2002; Iwakoshi et al. 2002; Iwakoshi-Ukena et al. 2004). Ir-GnRH was also observed in a cnidarian (Anctil 2000), a platyhelminthes (Anctil and Tekaya 2005) and a coral (Twan et al. 2006). As in vertebrates, it was demonstrated that GnRH serves multiple functions in reproduction or reproductive related activities, such as induction of spawning in a tunicate, *Ciona intestinalis* (Terakado 2001; Adams et al. 2003); a coral, *Euphyllia ancora* (Twan et al. 2006), and a chiton, *Mopalia* sp. (Gorbman et al. 2003); regulating gamete transport in an octopus, *O. vulgaris* (Di Cristo et al. 2002); stimulating rhythmic contraction of the octopus oviduct (Iwakoshi-Ukena et al. 2004); and acting as a pheromone in hemichordates, *Saccoglossus* and *Ptychodera* (Cameron et al. 1999) and *Mopalia* sp. (Gorbman et al. 2003). GnRH may also have a role in mediating neuronal signals not associated with reproduction, as a neurotransmitter and/or neuromodulator (Tsai 2006).

Recently, our group has demonstrated the presence of two GnRH-like peptides in CNS of the black tiger shrimp, *Penaeus monodon* (Ngernsoungnern et al. 2008). Both *M. rosenbergii* and *P. monodon* belong to the class Malacostraca and order Decapoda. However, at the suborder level, *M. rosenbergii* is classified as Pleocyemata, whereas *P. monodon* is a Dendrobranchiata (Burkenroad 1963). These two suborders are distinguished by their structure of the gills. The gills of the Dendrobranciata contain two series of branches, each member of a series being subdivided in a way that it appears brushy. All other decapods are assigned to the Pleocyemata which contains gills with either two broad lobes running the length of the central axis or various tiers of filaments (Kozloff 1990). To determine if GnRH or GnRH-like peptides are present in a freshwater prawn, we utilized various GnRH antisera in immunocytochemical studies. In the present study, we show the distribution of GnRH-like peptides in the CNS and ovary of the giant freshwater prawn, *M. rosenbergii.* This finding supports a hypothesis that GnRH or GnRH like peptides occur throughout various species of shrimp and perhaps other crustaceans.

Materials and methods

Animals

Mature female freshwater prawns, weighing between 30 and 40 g, were purchased from a local farm in Chonburi province, Thailand, and kept in a hatchery at Burapha University, Chonburi province for acclimatization for at least 2 weeks. They were maintained in concrete tanks (150×80 cm^2) containing fresh water with continuous aeration, under a natural photoperiod (12L:12D), at a temperature of about 25–30°C, and fed with commercial pellets twice daily.

Antisera and peptides

Five primary antisera used in this study consisted of anti-octopus (oct) GnRH, Lot. 9779 (generously provided by Dr. Pei-San Tsai), anti-salmon (s) GnRH, Lot. 1667 (a kind gift from Dr. Judy King), anti-lamprey (l) GnRH-I, Lot.1467 and anti-lGnRH-III, Lot. 3952, produced in the laboratory of Sower (1993), and anti-mammalian (m) GnRH (Sigma, St Louis, MO). The structures of peptides to which the antisera were raised are described in Table 1 (Gorbman and Sower 2003). Antiserum 9779 was selective for octGnRH, whereas approximately 50% immunoreactivity was abolished after preabsorbing the antisera with lGnRH-III, but not abolished after preabsorption with lGnRH-I (Ngernsoungnern et al. 2008). Antiserum 1667 demonstrated a specificity for sGnRH, but showed no cross-reactivity with lGnRH-III (Robinson et al. 2000). Antiserum 1467 has a specificity with cross-reactivities of 7.3% with lGnRH-III and less than 0.03, 0.02, and 0.01% cross-reactivity for chicken (c) GnRH-II, mGnRH, and cGnRH-I, respectively (Sower et al. 2000). Antiserum 3952 has cross-reactivities of 100% with lGnRH-I and III, less than 0.01% with mGnRH, cGnRH-I, cGnRH-II, and sGnRH (Robinson et al. 2000), and 0.1% with octGnRH (Ngernsoungnern et al. 2008). However, cross-reactivity of anti-mGnRH has not yet been identified in the previous study. To verify the specificities, anti-GnRHs were preabsorbed with lGnRH-I, lGnRH-III (American Peptide Company, Sunnyvale, CA), and octGnRH peptides (from Dr. Pei-San Tsai and Dr. Hiroyuki Minakata).

Immunocytochemistry

The prawn central nervous system (CNS) including eyestalk, supraesophageal, subesophageal, thoracic, and abdominal ganglia, and ovaries at spawn (stage 0), spent (stage 1), previttellogenic (stage 2), vittellogenic (stage 3) and mature stages (stage 4), as described by Meeratana and Sobhon (2007), were obtained from 10 adult female prawns, immediately fixed in Bouin's fixative overnight, and processed routinely for paraffin embedding. Serial sections were cut at 5 μm thick in horizontal plane, and then placed on poly-L-lysine coated slides. Immunoperoxidase technique used in this study was modified from the methods described by Sower et al. (1995). Briefly, the sections were deparaffinized, rehydrated, immersed in 70% ethanol

Table 1 The GnRH peptides to which the antisera were raised

Antiserum Lot.	Raised against	Amino acids of peptides positions										
		1			2	3	4	5	6	7	8	9	10
Unnamed	mGnRH	pGlu-			His-	Trp-	Ser-	Tyr-	Gly-	Leu-	Arg-	Pro-	Gly- NH2
1667	sGnRH	pGlu-			His-	Trp-	Ser-	Tyr-	Gly-	Trp-	Leu-	Pro-	Gly- NH2
1467	lGnRH-I	pGlu-			His-	Tyr-	Ser-	Leu-	Glu-	Trp-	Lys-	Pro-	Gly- NH2
3952	lGnRH-III	pGlu-			His-	Trp-	Ser-	His-	Asp-	Trp-	Lys-	Pro-	Gly- NH2
9779	octGnRH	pGlu-	Asn-	Tyr-	His-	Phe-	Ser-	Asn-	Gly-	Trp-	His-	Pro-	Gly- NH2

Data from Gorbman and Sower (2003)

containing 1% saturated $LiCO_3$ and 1% H_2O_2, and subsequently immersed in 0.1 M glycine and 0.1% Triton X-100 in order to get rid of picric acid, endogenous peroxidase, free aldehyde groups, and for tissue permeabilization, respectively. After blocking non-specific bindings with 4% bovine serum albumin (BSA), the sections were exposed to anti-mGnRH (1:20), anti-sGnRH (1:500), anti-octGnRH (1:500), anti-lGnRH-I (1:1,000), or anti-lGnRH-III (1:4,000) for overnight at 4°C. After extensive washing with phosphate buffer saline (PBS) containing 0.1% Tween-20 (PBST), the sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:500) (Zymed Laboratories, San Francisco, CA) for 60 min. Enzymatic reaction was developed by incubating the sections in 0.05% diaminobenzidine (DAB) and 0.03% H_2O_2. The sections were then dehydrated, cleared, mounted with mounting medium, observed under a Nikon ECLIPESE E600 light microscope, and images captured by Nikon ECIPESE 2000 CCD camera. The experiments were repeated at least three times for each tissue.

Immunofluorescence

The CNS and ovaries (as previously described) were immediately fixed in 4% paraformaldehyde in PBS overnight, extensively washed with PBS, followed by immersing in PBS containing 30% sucrose for overnight. The tissues were then embedded in O.C.T. compound (Miles Inc., Elkhart, IN), and serial cryosections were cut at 40 μm thick in horizontal plane and placed on poly-L-lysine coated slides. The cryosections were subsequently immersed in 0.1 M glycine and 0.1% Triton X-100, followed by blocking non-specific bindings with 4% BSA. The sections were then incubated with the same primary antisera at the same condition as previously described, and subsequently incubated with Alexa 488-conjugated goat anti-rabbit IgG (1:500) (Molecular Probes, Eugene, OR) for 60 min. In addition, the nuclei of cells in the CNS sections were stained with TO-PRO-3 (1:4,000) (Molecular Probes). The slides were viewed under an Olympus confocal laser scanning microscope (FV1000).

Specificities of antisera

The specificities of primary antisera were tested by preabsorption technique. Briefly, each of the primary antisera (100 μl at working dilution) was incubated with 100 μg of corresponding GnRH peptides, including octGnRH, sGnRH, lGnRH-I, and lGnRH-III for overnight, at 4°C. These preabsorbed antisera were then used in the immunoperoxidase and immunofluorescence staining instead of the non-absorbed antisera using the same conditions. The specificity of the secondary antiserum was performed by replacing the primary antisera with PBS.

Results

Immunocytochemistry

Five antisera (sGnRH, octGnRH, lGnRH-I, lGnRH-III and mGnRH) were used for detecting the existence of GnRHs in CNS and ovary of the prawn. Two ir-GnRHs (ir-lGnRH-III and ir-octGnRH) were detected in the CNS, whereas one ir-GnRH (ir-lGnRH-I) was observed in the ovary. The ir-lGnRH-III was detected in the soma of neurons located in deutocerebrum (Fig. 1a), within the neuronal cluster number 11 as classified by Sandeman et al. (1992) (Fig. 1c). All of the ir-lGnRH-III positive neuronal cell bodies belonged to medium-sized neurons with the diameter about 15 μm (Fig. 1a, inset). This result was confirmed by immunofluorescence which revealed the presence of the ir-lGnRH-III in the soma of medium-sized neuronal cells within the same cluster (Fig. 1d, e). More ir-lGnRH-III positive neuronal bodies were detected by the immunofluorescence when compared with those detected by the immunoperoxidase technique as a result of using thicker sections for detection of positive cells by confocal microscope, and the use of less denaturing conditions in immunofluorescence method. There was no ir-lGnRH-III observed in the sections incubated with preabsorbed anti-lGnRH-III (Fig. 1b, f). Interestingly, the ir-octGnRH was present only in the thoracic ganglia. Unlike the ir-lGnRH-

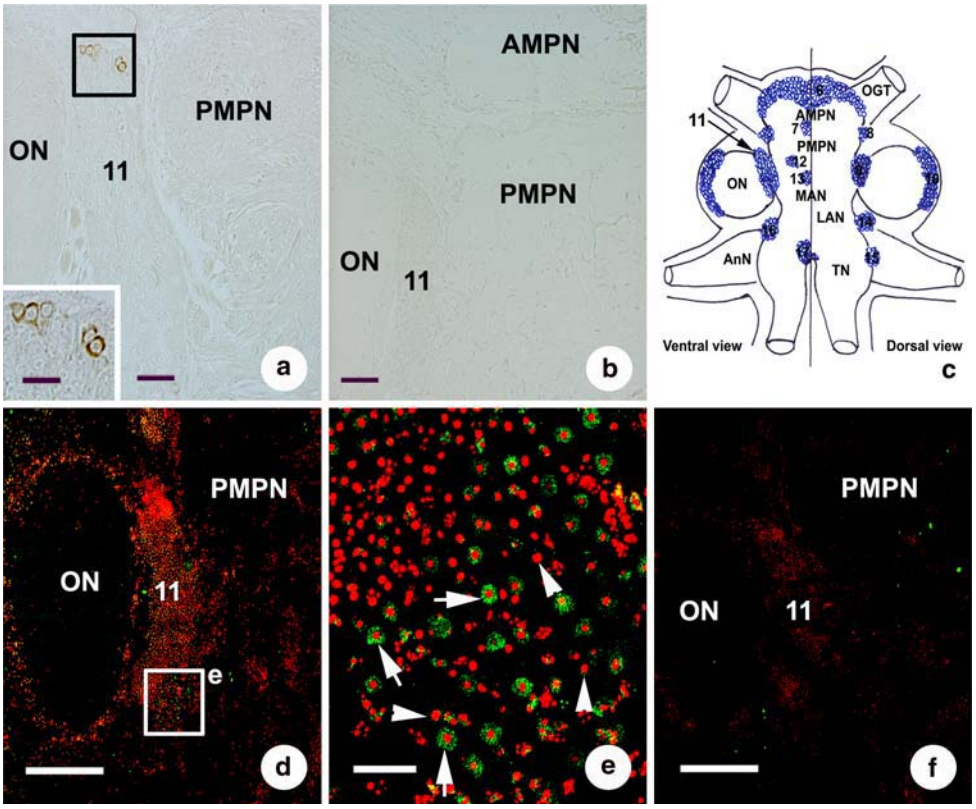

Fig. 1 Immunoperoxidase (**a**–**b**) and immunofluorescence (**d**–**f**), showing the distribution of ir-lGnRH-III in the prawn supraesophageal ganglion. **a** The ir-lGnRH-III is present in neuronal cell bodies within cluster 11 of the deutocerebrum. (**a**, inset) Higher magnification of boxed area in (**a**), showing the ir-lGnRH-III in neuronal cell bodies of medium-sized neurons. **b** Negative control section of immunoperoxidase, using lGnRH-III-preabsorbed antiserum as a probe, showing no ir-lGnRH-III. **c** A diagram showing the locations of neuronal cell clusters as numbered in the supraesophageal ganglion (modified from Sandeman et al. 1992). **d** Immunofluorescence showing the ir-lGnRH-III neuronal cell bodies in cluster 11. **e** Higher magnification of the *boxed area* in (**d**), revealing the ir-lGnRH-III in medium-sized neuronal cell bodies (*arrows*). There was no ir-lGnRH-III in some neuronal cells within the same cluster (*arrowheads*). **f** Negative control section of immunofluorescence using lGnRH-III-preabsorbed antiserum as a probe, showing no immunoreactivity. *AMPN* anterior medial protocerebral neuropil, *AnN* antenna II neuropil, *LAN* lateral antenna I neuropil, *MAN* medial antenna I neuropil, *PMPN* posterior medial protocerebral neuropil, *OGT* olfactory globular tract, *ON* olfactory neuropil, *TN* tegumental neuropil. Scale bars: 100 μm (**a** and **b**); 300 μm (**d** and **f**); 30 μm (**e**); 25 μm (**a**, inset)

III, the ir-octGnRH was not only detected in neuronal cell bodies, but was shown in the nerve fibers distributed in thoracic ganglia (Fig. 2). The ir-octGnRH was present in some medium-sized neurons located at dorsolateral neuronal cell cluster (DLC) (Fig. 2a, b, f), and some large-sized neurons (diameter of >25 μm) at ventromedial neuronal cell cluster (VMC) (data not shown). In addition, the ir-octGnRH fibers innervated some of the medium-sized and large-sized neuronal cells of the thoracic ganglion (Fig. 2c, d). This result was supported by immunofluorescence showing the existence of ir-octGnRH in the large-sized and medium-sized neuronal cells (Fig. 2g, h). In contrast, there were no ir-lGnRH-I, ir-sGnRH, and ir-mGnRH observed in any part of the prawn CNS. In the related control sections where anti-octGnRH was preabsorbed with octGnRH peptide, there was no immunostaining (Fig. 2e, i). The data of the distribution and intensities of ir-GnRHs in the prawn CNS are summarized in Table 2.

In the ovaries, the intense ir-lGnRH-I was detected in the cytoplasm of late previtellogenic oocyte (oocyte stage 2, Oc2) and early vitellogenic oocyte (oocyte stage 3, Oc3) as classified by Meeratana and Sobhon (2007) (Fig. 3a, b). In Oc2, the ir-lGnRH-I was observed at the perinuclear region and observed in the cytoplasm of Oc3 (Fig. 3b). The results obtained from immunofluorescence were consistent with the immunoperoxidase staining, showing the distribution of ir-lGnRH-I at the perinuclear region of the Oc2 and throughout the cytoplasm of the Oc3 (Fig. 3d, e). There was no ir-lGnRH-I observed in other areas of the ovary. In the control sections where anti-lGnRH-I was preabsorbed with lGnRH-I, there was no immunostaining (Fig. 3c, f). The detection of the ir-lGnRH-I in the prawn ovary is summarized in Table 3.

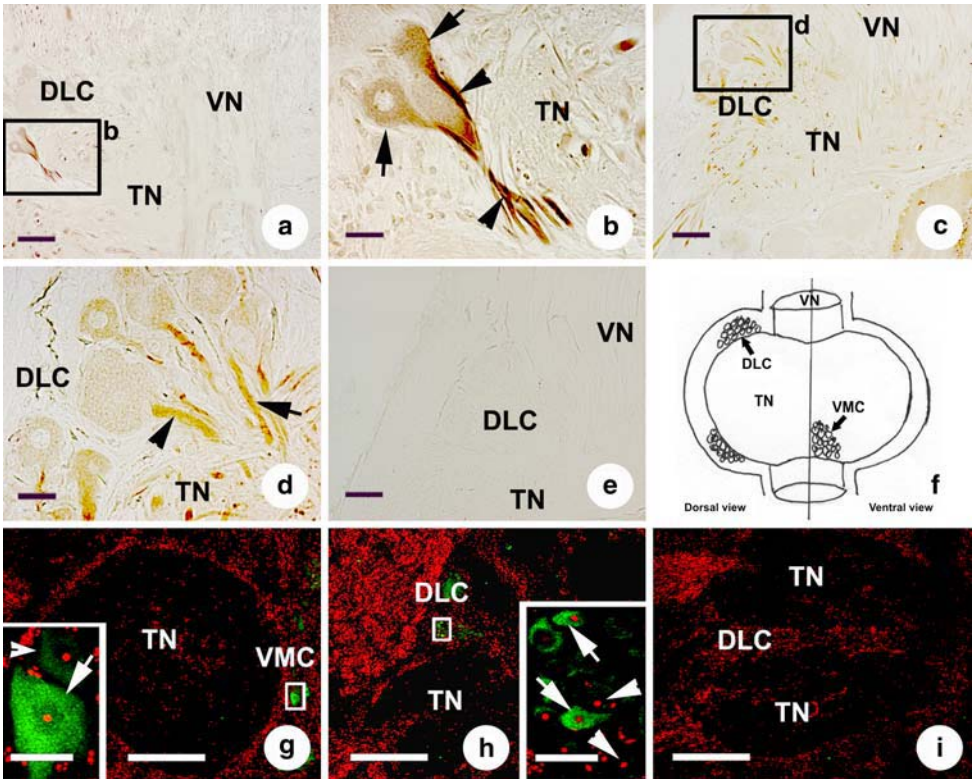

Fig. 2 Immunoperoxidase (**a–e**) and immunofluorescence (**g–i**), showing the distribution of ir-octGnRH in the prawn thoracic ganglia. **a** The ir-octGnRH is present in medium-sized neuronal cells in the dorsolateral neuronal cell cluster (DLC). **b** Higher magnification showing the ir-octGnRH in the soma of neuronal cells (*arrows*) and fibers (*arrowheads*) extending from the soma. **c** and **d** Light micrographs showing the ir-octGnRH positive fibers innervating medium-sized (**d**, *arrow*) and large-sized (**d**, *arrowhead*) neuronal cells. **e** Negative control using octGnRH-preabsorbed antiserum as a probe, revealing only background staining. **f** A diagram showing the locations of neuronal cell clusters in the thoracic ganglion. **g** and **h** Showing the ir-octGnRH in the soma of some large-sized neuronal cells in ventromedial neuronal cell cluster (VMC) (**g** and inset, *arrow*) and in medium-sized neuronal cells in DLC (**h** and inset, *arrows*). However, some neuronal cells within the same cluster show no ir-octGnRH (**g** and **h**, insets, *arrowheads*). **i** Negative control section of immunofluorescence using octGnRH-preabsorbed antiserum as a probe, showing only nuclear staining. *TN* thoracic neuropil, *VN* ventral nerve cord. Scale bars 100 μm (**a**, **c** and **e**); 25 μm (**b** and **d**); 300 μm (**g**, **h** and **i**); 30 μm (**g** and **h**, insets)

Antisera specificity

The immunoreactivities of anti-lGnRH-I, anti-lGnRH-III and anti-octGnRH were completely abolished when pre-absorbed with lGnRH-I, lGnRH-III and octGnRH peptides, respectively (Figs. 1b, f, 2e, i, 3c, f). The specificities of the five antisera are summarized in Table 4.

Discussion

In the present study, we demonstrated the presence of immunoreactive GnRH-like peptides in the CNS and ovary of the giant freshwater prawn. Interestingly, two of the five GnRH isoforms were detected in the neural tissue, whereas another one of the tested five GnRH isoforms was

Table 2 The distribution of ir-GnRHs in the CNS of *M. rosenbergii*

CNS parts	ir-lGnRH-III				ir-octGnRH			
	Neuronal cells[a]			Fibers	Neuronal cells[a]			Fibers
	Small	Medium	Large		Small	Medium	Large	
Eyestalk	–	–	–	–	–	–	–	–
Supraesophageal ganglion (neuronal cluster 11)	–	+ + +	–	–	–	–	–	–
Subesophageal ganglion	–	–	–	–	–	–	–	–
Thoracic ganglia	–	–	–	–	–	+ + +	+ + +	+ + +
Abdominal ganglia	–	–	–	–	–	–	–	–

–, no immunoreactivity; + + +, strong immunoreactivity

[a] Small neuronal cell, <15 μm in diameter; medium neuronal cell, 15–25 μm in diameter; large neuronal cell, >25 μm in diameter

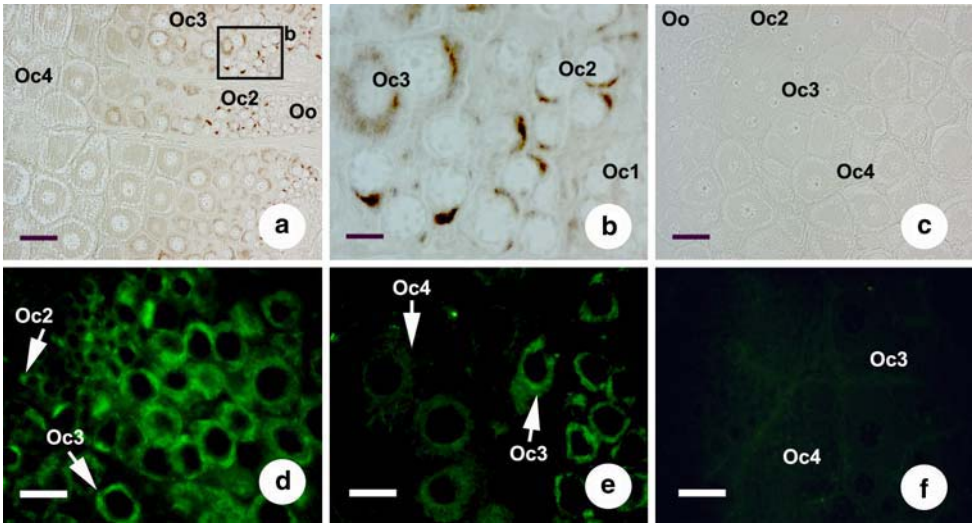

Fig. 3 Immunoperoxidase (**a–c**) and immmunofluorescence (**d–f**) showing the distribution of ir-lGnRH-I in the prawn ovary. The ir-lGnRH-I is present in Oc2 and Oc3 oocytes (**a** and **d**). Higher magnification showing the ir-lGnRH-I localized at the perinuclear region of Oc2 and in the cytoplasm of Oc3 (**b**, **d**, and **e**). Negative control sections of immunoperoxidase (**c**) and immunofluorescence (**f**) using lGnRH-I-preabsorbed antiserum as a probe, showing only background staining. *Oo* oogonia, *Oc2* oocyte stage 2 (late previtellogenic oocyte), *Oc3* oocyte stage 3 (early vitellogenic oocyte), *Oc4* oocyte stage 4 (late vitellogenic oocyte). Scale bars: 100 μm (**a** and **c**); 25 μm (**b**); 50 μm (**d**, **e** and **f**)

present in the ovary. These results suggest that there may be at least three GnRH-like peptides present in this prawn. The first GnRH isoform which was found in neuronal cell cluster 11 of the deutocerebrum was closely related to lGnRH-III. The second isoform which was found in soma and fibers of thoracic ganglia was closely related to octGnRH, and the third isoform observed in the oocytes was closely related to lGnRH-I. These results support previous findings that in one species of animal, either vertebrate or invertebrate, there are at lease two isoforms of GnRH (Fernald and White 1999; Gorbman and Sower 2003; Morgan and Millar 2004). The immunoreactivities of the anti-lGnRH-I and anti-lGnRH-III were observed in the different tissues of the prawn, i.e., ovary and CNS, respectively. In addition, the ir-octGnRH was also observed in the different parts of the prawn CNS compared to the ir-lGnRH-III. As shown in Table 1, these three GnRH isoforms are structurally different and that these differences are sufficient to have distinct epitopes that could generate antiserum-specific responses. The results from the preabsorption study further confirmed the specificity of the three antisera, because the immunoreactivities were completely abolished when the antisera were preabsorbed with corresponding GnRH peptides. These results suggested that there are at least three isoforms of GnRH in the prawn tissues. However, the primary structures of these GnRH-like peptides in the prawn might be similar but not identical to lGnRH-III, octGnRH, and lGnRH-I. Furthermore, these tissue-specific GnRH-like peptides could serve multiple functions including a possible role in reproduction.

In crustaceans, deutocerebrum is a major chemo-sensory organ that regulates various behaviors including feeding, locomotion, and mating (Sandeman et al. 1992). Similarly, in the aplysia, *A. californica*, ir-GnRH was detected in the osphradium, the major chemo-sensory structure of opisthobranch gastropod, and in CNS ganglia including cerebral, buccal, pedal, pleural, and abdominal (Tsai et al. 2003; Zhang et al. 2007) which regulate certain behaviors, including feeding, locomotion, copulation (Kandel 1979). In the present study, the ir-lGnRH-III neurons located in the prawn deutocerebrum may be involved in chemoreception that could be related indirectly to the control of reproductive behavior similar to the aplysia. However, the

Table 3 The distribution of ir-lGnRH-I in the ovary of *M. rosenbergii*

Ovarian follicles						
Oogonia	Early Previtellogenic oocyte	Late Previtellogenic oocyte	Early Vitellogenic oocyte	Late Vitellogenic oocyte	Mature Oocyte	Follicular cell
(Oo)	(Oc1)	(Oc2)	(Oc3)	(Oc4)	(Oc5)	
−	−	+ + +	+ + +	−	−	−

−, no immunoreactivity; + + +, strong immunoreactivity

Table 4 Specificities of the antisera tested with the preabsorption with various GnRH peptides

Antisera against	Immunoreactivity					
	Without preabsorption	Preabsorbed with				
		mGnRH	sGnRH	octGnRH	lGnRH-I	lGnRH-III
mGnRH (unnamed)	−	ND	ND	ND	ND	ND
sGnRH (1667)	−	ND	ND	ND	ND	ND
octGnRH (9779)	+ + +	ND	+ + +	−	+ + +	+ + +
lGnRH-I (1467)	+ + +	ND	+ + +	+ + +	−	+ + +
lGnRH-III (3952)	+ + +	ND	+ + +	+ + +	−	−

−, no immunoreactivity; + + +, strong immunoreactivity; *ND* not determined

exact function of GnRH-like peptide in deutocerebrum needs to be further elucidated. The finding that ir-octGnRH was present in neurons of thoracic ganglia and in the immunoreactive fibers innervating some neurons of the same ganglia suggest that the octGnRH-like peptide could be involved in regulating the release of certain neuroendocrine substance(s), such as gonad-stimulating hormone (GSH) which controls gonad maturation in prawn (Huberman 2000) and vitellogenenin synthesis in hepatopancreas (Van Herp and Payen 1991). Many studies have suggested that GSH is synthesized and excreted by neurosecretory cells in thoracic ganglia (Huberman 2000; Meeratana et al. 2006). Additionally, 5-hydroxytryptamine (5-HT) was also detected in the neurons of the freshwater prawn, *M. rosenbergii* thoracic ganglia (Sosa et al. 2004), and that the release of GSH could be induced by 5-HT (Fingerman 1997; Vaca and Alfaro 2000). Unfortunately, the relationship of GnRH and 5-HT has not yet been investigated in the freshwater prawn or related species. It is possible that the ir-octGnRH neurons observed in the present study may be involved in the prawn reproduction by stimulating the neurosecretory cells in thoracic ganglia to secrete GSH directly, or it could act in concert with 5-HT positive neurons. The co-localization of GnRH and 5-HT in the prawn CNS and the exact relationship between GnRH and 5-HT in stimulating the synthesis and release of GSH need more investigation before a firm conclusion can be reached.

In the present study, we demonstrated for the first time that a lGnRH-I-like peptide occurred in the prawn ovary. In vertebrates, it has been shown that GnRH was expressed in the ovary of human (Choi et al. 2006), a gold fish, *Carassius auratus* (Pati and Habibi 1998), and a rainbow trout, *Oncorhynchus mykiss* (Schalburg et al. 1999). In addition, GnRH receptors were also detected in the ovary of a goldfish, *C. auratus* (Pati and Habibi 1993), a common crap, *Cyprinus carpio* (Pati and Habibi 1992), and a mollusk, *O. vulgaris* (Kanda et al. 2006). These findings confirmed that a GnRH-like peptide exists in the fish ovaries, and that it may have paracrine/autocrine roles in the regulation of ovarian maturation (Pati and Habibi 1998). In a protochrodate, *C. intestinalis*, ir-mGnRH and ir-cGnRH-I were detected in the ovary, and it was further shown that these two isoforms of GnRH were involved in reproduction, including stimulating the synthesis and release of sex steroids from gonads, and the synthesis and release of luteinizing hormone from rat pituitary (Di Fiore et al. 2000). Tunicate (t) GnRH-III and l-GnRH-I were reported to induce gamete release from a chiton, *Mopalia* sp. (Gorbman et al. 2003). Moreover, in *A. californica*, the transcript for aplysia (ap) GnRH was expressed in the ovotestis, but its exact function has not yet been elucidated (Zhang et al. 2007). In the present study, the ir-lGnRH-I was detected at the perinuclear region of Oc2 and in cytoplasm of Oc3. It is possible that the lGnRH-I-like peptide was first synthesized in late previtellogenic oocytes (Oc2), accumulated in the cytoplasm of early vitellogenic oocytes (Oc3), and disappeared before these oocytes reached maturation. These findings suggest that in this prawn, lGnRH-I-like peptide may be directly involved in oocyte development. Similarly, GnRH was suggested to play a significant role in controlling of the oocyte maturation and spawning in a coral (Twan et al. 2006). The relationship between 5-HT and GnRH in the stimulation of oocyte maturation may also be possible as 5-HT was detected in the cytoplasm of the follicular cells and primary oocytes of *P. monodon* ovary. The ir-5-HT gradually increased in the cytoplasm of the oocytes in stage II to stage IV ovaries, and in addition to ovarian maturation, 5-HT was also shown to induce spawning in this shrimp (Wongprasert et al. 2006). Egg-laying hormone (ELH), a peptide, which is known to induce spawning in mollusks (Wayne 2001), was detected in the follicular cells surrounding oocytes of *Haliotis asinina* (Saitongdee et al. 2005) and *P. monodon*, thus it was also suggested that ELH may play a role in the induction of the ovarian maturation and spawning in shrimp (Liu et al. 2006). It is, therefore, possible that lGnRH-I-like peptide in the prawn ovary may act synergistically in some way with 5-HT and ELH in regulating the ovarian maturation and spawning of *P. monodon* as well as in this freshwater prawn. However, the

relationship of GnRH, 5-HT, and ELH should be explored by further research, particularly once the primary structure(s) of GnRH(s) are determined. An experiment determining the structure of the prawn GnRH is being attempted.

In conclusion, the present study demonstrated the presence of three isoforms of GnRH-like peptide in the CNS and ovarian tissues of *M. rosenbergii*. Two of these isoforms were closely related to ancient vertebrate GnRH peptides, namely lGnRH-I and lGnRH-III, whereas the other isoform was closely related to an invertebrate GnRH peptide, octGnRH. The three different isoforms of GnRH-like peptides are tissue specific and each may play different yet synergistic roles in the reproductive processes, i.e., neuromodulation and oocyte maturation, and/or chemosensory functions.

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The existence of gonadotropin-releasing hormone (GnRH) immunoreactivity in the ovary and the effects of GnRHs on the ovarian maturation in the black tiger shrimp *Penaeus monodon*

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ABSTRACT

Immunocytochemical localization using antibodies against five isoforms of gonadotropin-releasing hormone (GnRH), namely, luteinizing hormone-releasing hormone (LHRH), salmon (s)GnRH, octopus (oct)GnRH, lamprey (l)GnRH-I, and lGnRH-III, showed that only lGnRH-I immunoreactivity (ir-lGnRH-I) was localized in follicular cells of proliferative, vitellogenic, and mature ovaries. The effects of exogenous GnRHs on the ovarian maturation cycle of *Penaeus monodon* were compared by treating female broodstocks with LHRH, sGnRH, and lGnRH-I. The cycle of ovarian maturation in both eyestalk-ablated and eyestalk-intact shrimp administered with the three isoforms of GnRH was significantly shorter than that of the control animals. Moreover, administrations of all GnRH isoforms showed similar numbers of spawned eggs and the percentage of successful fertilization as in the control animals. These findings suggest that GnRHs may be highly conserved peptides that play an important role in inducing the ovarian maturation in the shrimp.

1. Introduction

In penaeid shrimp, the practical technique for the induction of ovarian maturation and spawning, is eyestalk ablation (Panouse, 1943). It is generally believed that reproduction-inhibiting factors, including gonad-inhibiting hormone (GIH) and mandibular organ-inhibiting hormone (MOIH), produced and secreted from the X-organ-sinus gland system in the eyestalk, are eliminated upon eyestalk ablation, thus the gonadal development is no longer inhibited (Huberman, 2000; Okumura, 2004). Although this method provides a reliable way to obtain oocyte maturation and spawning, it causes deterioration in egg quantity and quality over time, as well as limited use of the female broodstock (Vaca and Alfaro, 2000). Consequently, many alternative approaches have been proposed to replace eyestalk ablation, one of which is the endocrine manipulation. Attempts have been made to apply many reproductive-related factors identified in crustaceans, including neurotransmitters, peptide hormones, and steroids, in order to obviate eyestalk ablation. It has been reported that oocyte development of the sand shrimp, *Crangon crangon*, can be stimulated by human chorionic gonadotropin (HCG) (Bomirski and Klek-Kawinska, 1976). Similar effect has been obtained using terpenoid hormones including methyl farnesoate (MF) or juvenile hormone-III (JH-III), which could stimulate ovarian maturation and increase oocyte diameters in *P. vannamei* (Tsukimura and Kamemoto, 1991). Furthermore, one of the well-known neurotransmitter, 5-hydroxytryptamine (5-HT), has been shown to stimulate ovarian maturation in *Procambarus clarkii* (Sarojini et al., 1995), *P. vannamei* (Vaca and Alfaro, 2000), *Macrobrachium rosenbergii* (Meeratana et al., 2006), and also in *P. monodon* (Wongprasert et al., 2006). However, at present the diversity of hormones involved in shrimp reproduction, their individual roles and relatedness, have not yet been fully defined. A better understanding of shrimp endocrinology is essential for hormonal manipulation to be applied in shrimp aquaculture.

GnRH, a well characterized peptide hormone, has long been known to function in regulating gonadal development and reproduction in both vertebrates and invertebrates (Sherwood et al., 1993; Fernald and White, 1999; Gorbman and Sower, 2003; Tsai, 2006). Presently, GnRH is characterized into 25 isoforms: 14 isoforms in vertebrates and 11 isoforms in invertebrates. All these isoforms are classified into 5 major groups: GnRH1, GnRH2, GnRH3, GnRH4, and GnRH5, based on phylogenetic analysis, function, distribution, and developmental origin (Silver et al., 2004; Sower et al., 2004; Zhang et al., 2008). The existence of GnRH immunoreactivity (ir-GnRH) had been demonstrated in

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various tissues of invertebrate species, for examples, neural tissue of mollusks (Goldberg et al., 1993; Di Cosmo and Di Cristo, 1998; Pazos and Mathieu, 1999; 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; Zhang et al., 2008), and gonadal tissue of mollusks (Young et al., 1999; Zhang et al., 2000), and a tunicate (Tsutsui et al., 1998; Di Fiore et al., 2000). In addition, the reproductive-related function of GnRH had been demonstrated in a number of invertebrate species, including a tunicate, *Ciona intestinalis* (Terakado, 2001; Adams et al., 2003), mollusks, *Octopus vulgaris*, *Mopalia* sp., and *Aplysia californica* (Iwakoshi et al., 2002; Gorbman et al., 2003; Tsai et al., 2003; Iwakoshi-Ukena et al., 2004), and a coral, *Euphyllia ancora* (Twan et al., 2006). The reproductive function of GnRH has also been indirectly demonstrated in *P. monodon* by the ability of its hepatopancreatic extract in inducing LH release from rat anterior pituitary glands *in vitro* (Fann et al., 1990). Our research group has recently demonstrated the existence of GnRH-like factors in *P. monodon* by detecting the presence of immunoreactivities against lGnRH-III and octGnRH in neurons of the supraesophageal ganglion and some nerve tracts of *P. monodon* CNS (Ngernsoungnern et al., 2008b). Nevertheless, the role of GnRH in shrimp reproduction is still not yet understood. We demonstrate, herein, the presence of a GnRH-like factor in the ovary of *P. monodon*, and the effect of three GnRH isoforms on enhancing the ovarian maturation in this species.

2. Materials and methods

2.1. Animals

Male and female *P. monodon* broodstocks weighing about 120–180 g and 200–280 g, respectively, were caught from Andaman Sea, and subsequently acclimatized for at least one week at a commercial farm in Rayong Province, Thailand. The shrimp were maintained in concrete tanks containing filtered seawater with ~30 ppt salinity, at 25–28 °C, and continuous aeration. The male and female shrimp were kept together at the ratio, 1:3 to allow natural mating. The shrimp were fed twice a day with minced squids. Approximately 70% of seawater was replaced daily. Unilateral eyestalk ablation was performed on one group of the female shrimp.

2.2. Antibodies and peptides

For immunocytochemistry, five available antibodies against GnRHs were used. Because the shrimp is considered to be a protostome, the first antibody chosen had been the antibody raised against octGnRH (anti-octGnRH, Lot 9779, generously provided by Dr. Pei-San Tsai). The other three antibodies were generated against GnRH isoforms of one lower vertebrate (lamprey) and one higher vertebrate (salmon). These three antibodies included anti-sGnRH, Lot 1667 (a gift from Dr. Judy King), anti-lGnRH-I, Lot 1467, and anti-lGnRH-III, Lot 3952 (gifts from Dr. Stacia A. Sower). Moreover, anti-LHRH (Sigma, St. Louis, MO) was also used to determine the presence of LHRH, which may be similar to higher vertebrate GnRH, in the shrimp tissue. For preabsorption and bioassay studies, lGnRH-I (generously provided by Dr. Stacia A. Sower), sGnRH and LHRH (Sigma) were used.

2.3. Immunocytochemistry for detecting GnRH-immunoreactivity in the ovary

The ovaries from shrimp at different stages of ovarian maturation (proliferative, vitellogenic, and mature) were carefully removed and fixed in Bouin's fixative overnight. The tissue blocks were then dehydrated with increasing concentrations of ethanol, cleared in dioxane, and further processed for routine paraffin embedding. Five-micron thick sections were deparaffinized, rehydrated, and immersed in 1% H_2O_2 and 1% $LiCO_3$ in 70% ethanol to eliminate endogenous peroxidase and residual picric acid, respectively. Free aldehydes in the tissue were minimized by immersing slides in 0.1 M glycine in phospahate buffered saline (PBS). Tissue was permeabilized with 1% Triton X-100 in PBS, and non-specific binding was blocked with 4% BSA in PBS. The sections were then incubated with anti-LHRH (dilution 1:40), anti-sGnRH (1:500), anti-lGnRH-I (1:1000), anti-lGnRH-III (1:4000), or anti-octGnRH (1:500) antibodies overnight at 4 °C. The negative controls were done by omitting the primary antibodies in the initial incubation step or incubating tissue sections in primary antibodies preabsorbed with the corresponding GnRH peptides (50 μg/0.1 mL antisera at the working dilutions). The sections were washed extensively with PBS containing 0.1% Tween-20, and subsequently exposed to peroxidase-conjugated goat anti-rabbit IgG (Sigma) at a 1:1500 dilution for 1 h at room temperature. The antigen-antibody complexes, indicating the existence of GnRH-liked factor, were visualized by incubating the sections with diaminobenzidine (DAB) enhanced liquid substrate system (Sigma). The sections were then dehydrated, cleared, and mounted with mounting reagent prior to observation under a Nikon Eclipse light microscope equipped with a Nikon DXM 1200 CCD camera.

Since anti-lGnRH-I was the only antibody that showed positive results in immunoperoxidase staining, an immunofluorescence technique was also performed to confirm the result in ovarian sections using anti-lGnRH-I (1:1000) under the same conditions as previously described for immunoperoxidase technique, except the corresponding secondary antibody was Alexa-488 conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) diluted at 1:300. The fluorescent micrographs were captured by the same Nikon microscope.

2.4. GnRH administrations to stimulate ovarian maturation

Three isoforms of GnRH used to stimulate ovarian maturation in this study included LHRH, lGnRH-I and sGnRH. This was based on the reports which showed that LHRH was used to stimulate gonadal maturation and spawning in a shrimp, *P. semisulcatus* (Aktas, 2005), whereas lGnRH-I was reported to have an effect on gamete release in a protochordate, *Ciona intestinalis* (Gorbman et al., 2003). Each isoform of GnRHs was diluted in normal saline (NS) to a desired dosage immediately before administration. The unilateral eyestalk-ablated female shrimp were divided into 6 groups ($n=7$ each), and designated as follows: 1) NS injection which served as the control group; 2) and 3) sGnRH injections at 10 and 50 ng/g body weight (BW), respectively; 4) and 5) LHRH injections at 10 and 50 ng/g BW, respectively; and 6) lGnRH-I injection at 50 ng/g BW. Because the preliminary result showed that the low dose of LHRH and sGnRH (10 ng/g BW) had no effect on shortening the duration of ovarian maturation, and because its availability was limited, the low dose of lGnRH-I was not performed. And because of its limited availability, octGnRH was not used in this experiment. Approximately 0.1 mL of NS or diluted GnRHs was injected into the shrimp dorsal muscles between the 1st and 2nd thoracoabdominal segments. Hormonal injections were carried out on day 1 after spawning. Stages of ovarian development following the hormonal administration were checked daily by flashing the light through thoracoabdominal segments of the shrimp to confirm the ovarian stages as described in the Results.

Another experiment was also conducted on non-ablated female broodstocks. Because of a limited number of these shrimp, the dose of GnRH used in this experiment was greater than that used in a report of Aktas (2005), which showed that GnRH at the 200 ng/g BW did not show marked changes in gonadal development and spawning. We, therefore, decided to increase the dose by 2.5 fold to 500 ng/g BW of GnRHs in this experiment, which was 10 times higher than the highest dose tested with the eyestalk-ablated shrimp. The non-ablated shrimp were divided into 3 groups ($n=7$ each) as follows: 1) NS injection which served as the control group; 2) sGnRH injection at 500 ng/g BW; and 3) lGnRH-I injection at 500 ng/g BW. Thereafter, the shrimp were