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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 supra-esophageal 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-

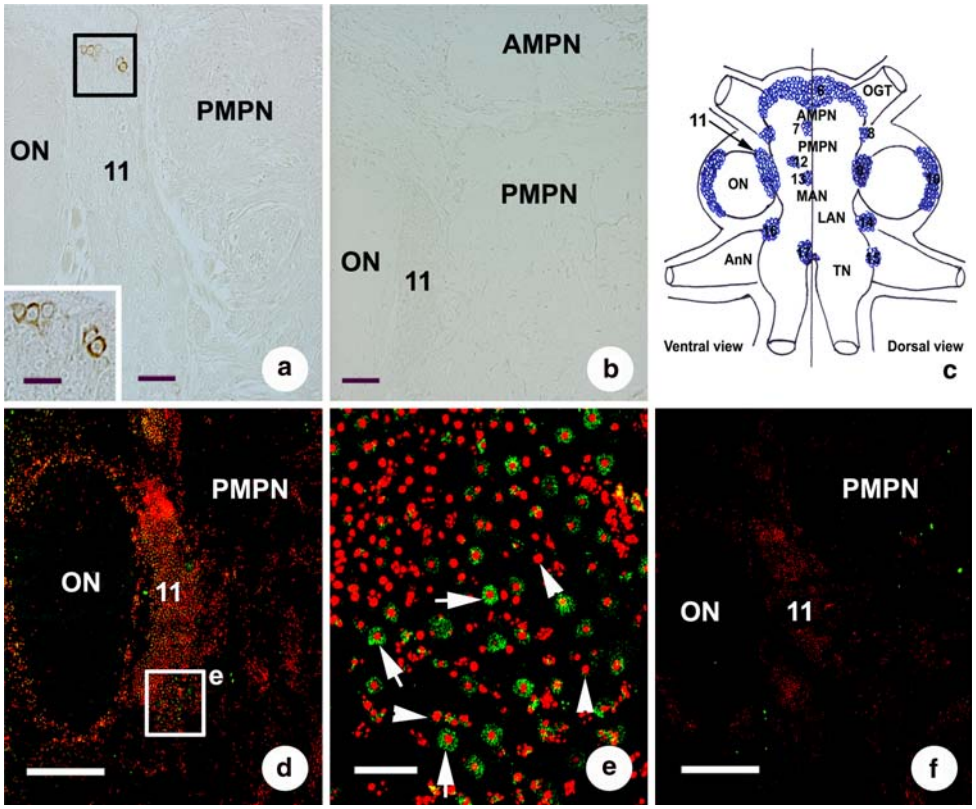

Fig. 1 Immunoperoxidase (**a**–**b**) and immunofluorescence (**d**–**f**), showing the distribution of ir-lGnRH-III in the prawn supraesophageal ganglion. **a** The ir-lGnRH-III is present in neuronal cell bodies within cluster 11 of the deutocerebrum. (**a**, inset) Higher magnification of boxed area 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.

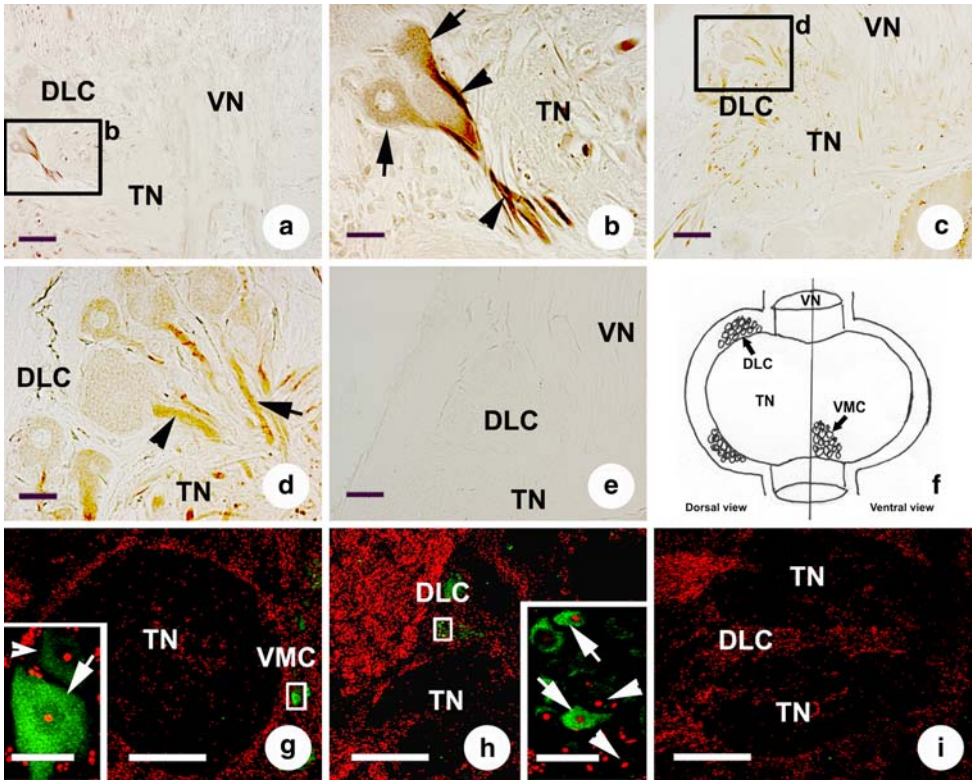

Fig. 2 Immunoperoxidase (**a–e**) and immunofluorescence (**g–i**), showing the distribution of ir-octGnRH in the prawn thoracic ganglia. **a** The ir-octGnRH is present in medium-sized neuronal cells in the dorsolateral neuronal cell cluster (DLC). **b** Higher magnification showing the ir-octGnRH in 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

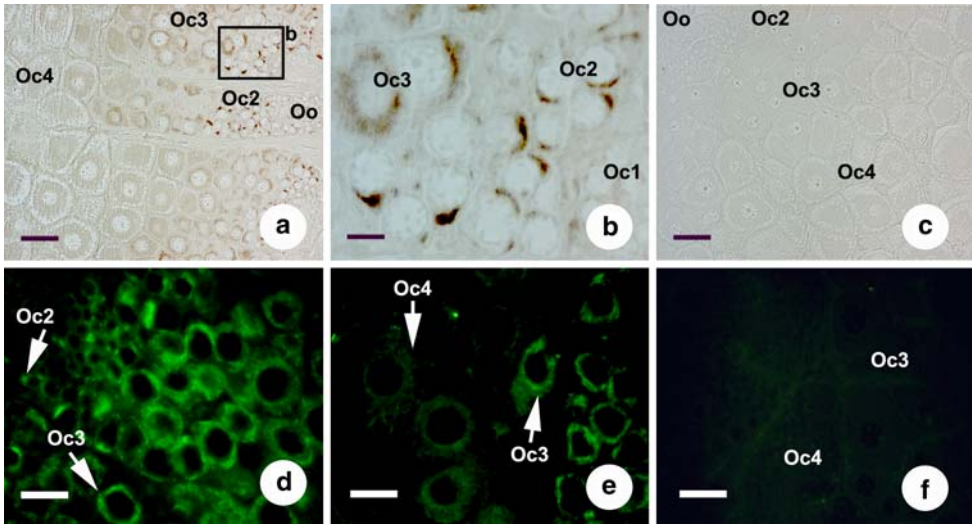

Fig. 3 Immunoperoxidase (**a–c**) and immmunofluorescence (**d–f**) showing the distribution of ir-lGnRH-I in the prawn ovary. The ir-lGnRH-I is present in Oc2 and Oc3 oocytes (**a** and **d**). Higher magnification showing the ir-lGnRH-I localized at the perinuclear region of Oc2 and in the cytoplasm 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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