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Source: Zoological Science, 26(12) : 840-845

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.26.840

ZOOLOGICAL SCIENCE **26**: 840–845 (2009)

Biochemical Analysis and Immunohistochemical Examination of a GnRH-like Immunoreactive Peptide in the Central Nervous System of a Decapod Crustacean, the Kuruma Prawn (*Marsupenaeus japonicus*)

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We examined whether a gonadotropin-releasing hormone (GnRH)-like peptide exists in the central nervous system (CNS) of the kuruma prawn, *Marsupenaeus japonicus*, by reverse-phase high performance liquid chromatography (rpHPLC) combined with time-resolved fluoroimmunoassay (TR-FIA) analysis and by immunohistochemistry. The displacement curve obtained for serially diluted extracts of the kuruma prawn brain paralleled the chicken GnRH-II (cGnRH-II) standard curve obtained by cGnRH-II TR-FIA using the anti-cGnRH-II antibody, which cross-reacts not only with cGnRH-II but also with lamprey GnRH-II (lGnRH-II) and octopus GnRH (octGnRH). Extracts of kuruma prawn brains and eyestalks showed a similar retention time to synthetic lGnRH-II and octGnRH in rpHPLC combined with TR-FIA analysis. Using this antibody, we detected GnRH-like-immunoreactive (ir) cell bodies in the anterior-most part of the supraesophageal ganglion (brain), the protocerebrum. Furthermore, GnRH-like-ir fibers were observed in the protocerebrum and deutocerebrum. In the eyestalk, GnRH-like-ir cell bodies were detected in the medulla interna, and GnRH-like-ir fibers were distributed in the medulla interna, medulla externa, and lamina ganglionalis. In the thoracic ganglion, GnRH-like-ir fibers, but not GnRH-like-ir cell bodies, were detected. No GnRH-like-ir cell bodies or fibers were detected in the abdominal ganglion or ovary. Thus, we have shown the existence and distribution of a GnRH-like peptide in the CNS of the kuruma prawn.

Key words: GnRH neuron, brain, eyestalk, immunohistochemistry, HPLC, invertebrate

INTRODUCTION

Gonadal maturation in vertebrates is primarily regulated by the brain-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH) stimulates the synthesis and release of pituitary gonadotropin (GTH), which stimulates the secretion of steroid hormones from the gonads. Recent studies have shown that two or three molecular forms of GnRH exist even within the same species (Oka, 1997; Okuzawa and Kobayashi, 1999; Okubo and Nagahama, 2008).

In addition to the peptides present in vertebrate species, GnRH peptides have been isolated and their sequences determined in the protochordates *Chelyosoma productum* (Powell et al., 1996) and *Ciona intestinalis* (Adams et al., 2003), and the octopus *Octopus vulgaris* (Iwakoshi et al., 2002). Moreover, the full-length cDNA of a GnRH-like molecule was isolated from the central nervous system (CNS) of a gastropod mollusk, the sea hare *Aplysia californica* (Zhang et al., 2008). Furthermore, the existence of GnRH-like immunoreactive (ir) materials has been indicated by using immunohistochemistry and/or biochemical techniques in various invertebrates (see Gorbman ans Sower, 2003; Kah et al., 2007; Pierantoni et al., 2002; Rastogi et al., 2002; Tsai, 2006). Recently, in a decapod crustacean, the black tiger shrimp (*Penaeus monodon*), octopus GnRH (oct-GnRH)-like-ir and lamprey GnRH-III (lGnRH-III)-like-ir cell bodies were detected in the supraesophageal ganglion (brain), octGnRH-like-ir fibers were detected in the brain and segmental ganglia (subesophageal, thoracic, and abdominal ganglia), and lGnRH-I-like immunoreactivity was detected in the ovary (Ngernsoungnern A et al., 2008a; Ngernsoungnern P et al., 2008). Moreover, in the giant freshwater prawn *Macrobrachium rosenbergii*, lGnRH-III-like-ir cell bodies were detected in the brain, octGnRH-like-ir cell bodies and fibers were detected in the thoracic ganglion, and lGnRH-I-like immunoreactivity was detected in the ovary (Ngernsoungnern

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doi:10.2108/zsj.26.840

A et al., 2008b). Interestingly, the GnRH forms present in the brains of these two crustaceans are different, and GnRH immunoreactivity was not detected in the eyestalk, where a number of neurohormones are synthesized.

In this study, we examined whether a GnRH-like peptide exists in the CNS of the decapod crustacean, the kuruma prawn (*Marsupenaeus japonicas*), which is the most commercially important species in shrimp aquaculture in Japan, by reverse-phase high performance liquid chromatography (rpHPLC) combined with time-resolved fluoroimmunoassay (TR-FIA) analysis and by immunohistochemistry. A preliminary experiment showed that a rabbit polyclonal antibody (aCII6) raised against chicken GnRH-II (cGnRH-II) immunostained the kuruma prawn brain.

MATERIALS AND METHODS

Kuruma prawns

Subadult (immature prepuberal) female kuruma prawns were purchased from a local fish farm in Okinawa Prefecture, Japan. Their body weights ranged from approximately 20 to 60 g.

TR-FIA

First, we examined whether a GnRH-like peptide exists in the kuruma prawn brain by performing a TR-FIA for cGnRH-II using aCII6 (Amano et al., 2004). Senthilkumaran et al. (1999) previously reported the cross-reactivities of aCII6 against salmon GnRH (sGnRH), seabream GnRH, lGnRH-I, mammalian GnRH (mGnRH), cGnRH-I, and catfish GnRH. In this study, we further examined the cross-reactivities of aCII6 against tunicate GnRH-I (tGnRH-I), tGnRH-II, lGnRH-II, and lGnRH-III, dogfish GnRH, herring GnRH, medaka GnRH (pejerrey GnRH), whitefish GnRH, frog GnRH, guinea pig GnRH, and octGnRH. The cross-reactivities of aCII6 against lGnRH-II (41.8%) and octGnRH (47.8%) were relatively high, as summarized in Table 1.

The kuruma prawns were anesthetized in ice. Their brains were rapidly dissected, frozen in dry ice, and stored at –85°C until analysis. GnRH was extracted according to a previously reported method (Amano et al., 2004). Parallelism between the typical standard curve for cGnRH-II and the competition curves obtained for the kuruma prawn brain extracts was examined by using a 2-fold serially diluted standard and the brain extracts in an assay buffer (Amano et al., 2008).

Table 1. Cross-reactivites of aCII6 agaist various forms of GnRH. Cross-reactivity was measured at B/B_0=50%. * According to Senthilkumaran et al. (1999).

GnRH forms		Cross-reactivity (%)
		anti-cGnRH-II (aCII6)
Mammalian GnRH	(mGnRH)	*<0.01
Guinea pig GnRH	(gpGnRH)	0.05
Chicken GnRH-I	(cGnRH-I)	*<0.01
Chicken GnRH-II	(cGnRH-II)	100
Frog GnRH	(frGnRH)	0.03
Salmon GnRH	(sGnRH)	*1.46
Catfish GnRH	(cfGnRH)	*<0.01
Seabream GnRH	(sbGnRH)	*<0.01
Medaka GnRH	(mdGnRH)	<0.01
Herring GnRH	(hrGnRH)	<0.01
Dogfish GnRH	(dfGnRH)	0.52
Whitefish GnRH	(wfGnRH)	<0.01
Lamprey GnRH-I	(lGnRH-I)	*<0.01
Lamprey GnRH-II	(lGnRH-II)	41.8
Lamprey GnRH-III	(lGnRH-III)	<0.01
Tunicate GnRH-I	(tGnRH-I)	0.23
Tunicate GnRH-II	(tGnRH-II)	<0.01
Octopus GnRH	(octGnRH)	47.8

rpHPLC combined with TR-FIA

For rpHPLC combined with TR-FIA analysis, 66 brains and 34 eyestalks (17 individuals) were extracted according to the method of Okuzawa et al. (1990), with slight modification. Frozen brain ganglion and eyestalk tissues were homogenized in 100:3 acetone : 1N HCl (v/v; 5 ml/g frozen tissue). The homogenate was stirred for 3 hr on dry ice and then centrifuged at 10,000×*g* for 30 min at 4°C. The precipitate was re-extracted in 80:20 acetone : 0.01 N HCl (v/v) in two-fifths the volume of the original extraction fluid, stirred for 5 min, and re-centrifuged. The combined supernatant was then extracted with petroleum ether to remove the acetone, lipids, and other hydrophobic substances. The ratio of filtrate (or aqueous phase) to petroleum ether was maintained at approximately 4:1 (v/v) for each of five successive extractions. The final aqueous phase was passed through a 0.22-μm filter (Millipore, Bedford, MA).

Since aCII6 also cross-reacts with lGnRH-II and octGnRH (Table 1), the extracts of the brain and eyestalk were separated by rpHPLC under the same conditions used to separate cGnRH-II, lGnRH-II, and octGnRH, as described below. The filtrate (800–900 μl) was injected through a 1-ml injection loop onto an Asahipak Gel ODP-50 column (0.46×25 cm; Asahi Chemical Industry, Kawasaki, Japan). Liquid chromatography was performed using a flow rate of 1 ml/minute and a high-pressure gradient system with two pumps (880-PU, JASCO, Tokyo, Japan) with 20 mM triethyamine acetate, pH 7.0 (TEAA) and acetonitrile containing 16 mM TEAA. The percentage of acetonitrile was increased linearly from 10% (0 min) to 50% (50 min). Fractions were collected every minute. The standard solution consisting of 1 μg of cGnRH-II, lGnRH-II, and octGnRH in 200 μl of 0.1% trifluoroacetic acid was injected and fractionated as explained above. The fractions collected from the brain and eyestalk samples were freeze dried by using a vacuum centrifuge concentrator. The respective dried residues were re-dissolved in 200 μl of TR-FIA assay buffer (20 mM sodium phosphate buffer, 0.9% NaCl, 0.1% BSA, 20 μM diethylenetriamine-N,N,N',N",N"-pentaacetic acid, 0.01% Tween-40, pH 7.2) and were assayed for cGnRH-II by TR-FIA (Amano et al., 2004).

Immunohistochemistry

For immunohistochemistry, the brain, eyestalk, thoracic ganglion, and abdominal ganglion were fixed in Bouin's fluid for 24 hours at 4°C and subsequently rinsed in cold 70% ethanol, dehydrated through a graded series of ethanol concentrations, and embedded in Paraplast (Monoject, Sherwood Medical, St Louis, MO). Sagittal and frontal sections were cut at 8 μm and mounted on gelatinized slides. Immunohistochemistry tests were conducted according to the method of Amano et al. using aCII6 (Amano et al., 2008). A Histofine immunostaining kit (Nichirei, Tokyo, Japan) was used for the immunohistochemical reactions. The sections were counterstained with Mayer's hematoxylin. To test the specificity of the immunoreactions, control sections were incubated with aCII6 that had been preabsorbed overnight at 4°C with an excess of cGnRH-II, lGnRH-II or octGnRH (10 μg cGnRH-II, lGnRH-II, or octGnRH in 1 ml antiserum). The subsequent procedure was identical to that used for the experimental sections. We followed the terminology for the neuronal cluster devised by Sandeman et al. (1992).

RESULTS

TR-FIA

The displacement curve obtained for the serially diluted extracts of the kuruma prawn brain paralleled the cGnRH-II standard curve (Fig. 1).

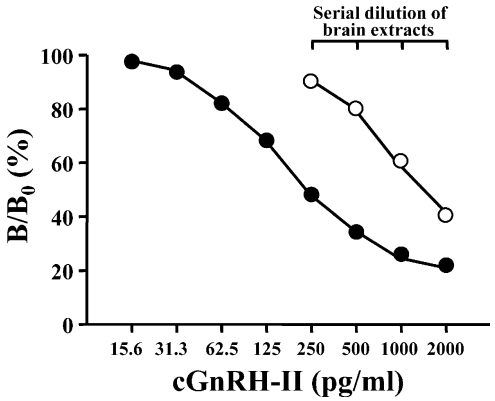

Fig. 1. A typical cGnRH-II standard curve and a competition curve for 2-fold serially diluted kuruma prawn brain extracts.

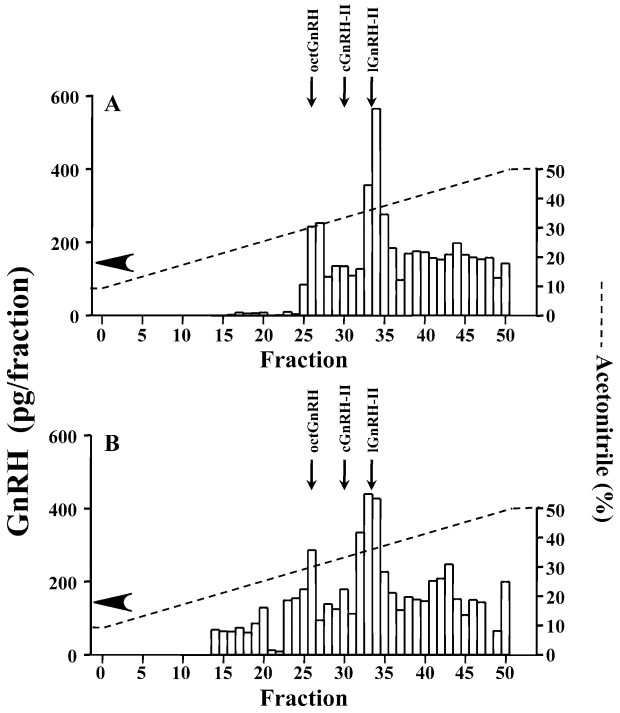

Fig. 2. Reverse-phase HPLC of **(A)** kuruma prawn brain and **(B)** eyestalk extracts followed by cGnRH-II TR-FIA. The arrows indicate the elution times of synthetic octGnRH, cGnRH-II, and lGnRH-II, and the arrowheads indicate the minimum detectable limit of cGnRH-II TR-FIA. The mobile phase consisted of CH_3CN (acetonitrile) containing 16 mM TEAA. The dotted lines represent the percentage of acetonitrile in the mobile phase.

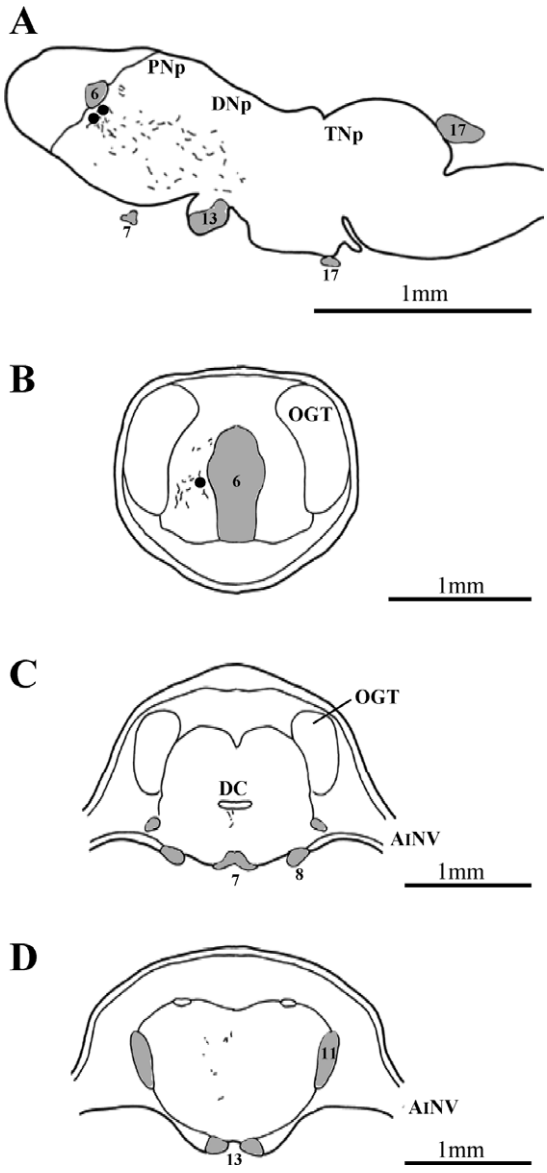

Fig. 3. (A) Schematic illustration of GnRH-like-ir cell bodies (closed circles) and fibers in a parasagittal section of the kuruma prawn. The rostral side is on the left. **(B–D)** Schematic illustration of GnRH-like-ir cell bodies (closed circles) and fibers in frontal sections of the kuruma prawn brain from the rostral **(B)** to the caudal **(D)** ends. Dorsal is at the top. A_lN_V, antenna I nerve; DC, deutocerebral commissure; DNp, deutocerebral neuropil; OGT, olfactory globular tract; PNp, protocerebral neuropil; TNp, tritocerebral neuropil. Shaded areas and numbers indicate neuronal clusters and their numbers according to the classification by Sandeman et al. (1992).

rpHPLC combined with TR-FIA

The kuruma prawn brain and eyestalk extracts showed a similar retention time to synthetic lGnRH-II and octGnRH in rpHPLC combined with TR-FIA analysis, but did not show a similar retention time to cGnRH-II (Fig. 2).

Immunohistochemistry

The distribution of GnRH-like-ir cell bodies and fibers in the parasagittal and frontal sections of the brain is depicted in Fig. 3. The GnRH-like-ir cell bodies were detected in the anterior-most part of the brain, the protocerebrum. They were located in close proximity to but more caudal than the medium-sized neurons of neuronal cluster 6, which are located in the anterior-most part of the shrimp brain (Figs. 3A, B, 4A–G). The GnRH-like-ir cell bodies measured 11.2±1.1 μm (n=10). GnRH-like-ir fibers were observed in the protocerebrum and deutocerebrum (Figs. 3A–D, 4A–G). No GnRH-like immunoreactivity was observed when the antiserum was preabsorbed overnight at 4°C with an excess

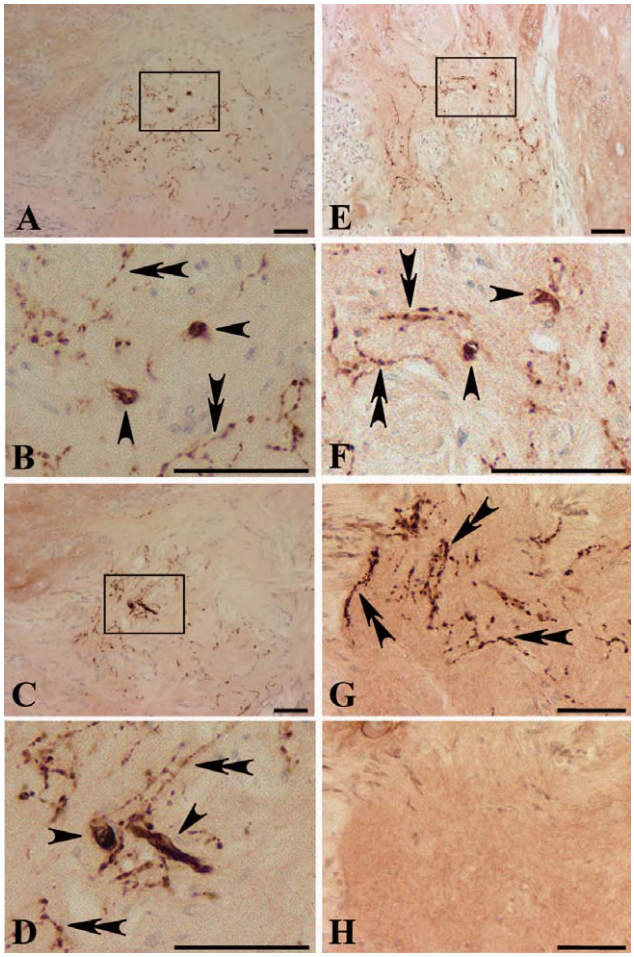

Fig. 4. **(A)** Sagittal section through the brain incubated with aCII6. The rostral side is to the left. **(B)** The boxed area in (A) at a higher magnification. **(C)** Sagittal section through the brain incubated with aCII6. The rostral side is to the left. **(D)** The boxed area in (C) at a higher magnification. **(E)** Frontal section through the brain incubated with aCII6. Dorsal is at the top. **(F)** The boxed area in (E) at a higher magnification. **(G)** Sagittal section through the brain incubated with aCII6. The rostral side is to the left. **(H)** Section adjacent to (G) incubated with aCII6 preabsorbed with cGnRH-II. Arrowheads and double arrowheads indicate GnRH-like-ir cell bodies and fibers, respectively. Scale bars: 50 μm.

of cGnRH-II (Fig. 3H), lGnRH-II, and octGnRH (data not shown).

In the eyestalk, GnRH-like-ir cell bodies were detected in the medulla interna (Fig. 5A, D–F). The GnRH-like-ir cell bodies measured 4.4±0.2 μm (n=10). A large number of GnRH-like-ir fibers were detected in the medulla interna (Fig. 5A, B, E) and lamina ganglionalis (Fig. 5C), and a few GnRH-like-ir fibers were observed in the medulla externa (Fig. 5D). No GnRH-like immunoreactivity was observed when the antiserum was preabsorbed overnight at 4°C with an excess amount of cGnRH-II (Fig. 5G), lGnRH-II, and octGnRH (data not shown).

In the thoracic ganglion, GnRH-like-ir fibers, but not GnRH-like-ir cell bodies, were detected in the neuropil (Fig. 6A–C). No GnRH-like immunoreactivity was observed when the antiserum was preabsorbed overnight at 4°C with an

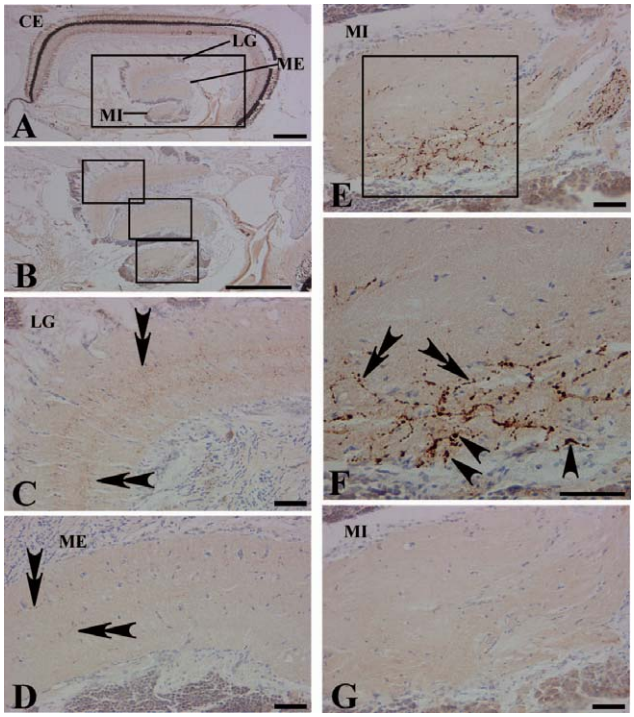

Fig. 5. **(A)** Sagittal section through the eyestalk incubated with aCII6. The rostral side is to the left. **(B)** The boxed area in (A) at a higher magnification. **(C)** The boxed area in "B" (LG) at a higher magnification. **(D)** The boxed area in (B) (ME) at a higher magnification. **(E)** The boxed area in (B) (MI) at a higher magnification. **(F)** The boxed area in (E) (MI) at a higher magnification. **(G)** Section adjacent to (E) incubated with aCII6 preabsorbed with cGnRH-II. Arrowheads and double arrowheads indicate GnRH-like-ir cell bodies and fibers, respectively. CE, compound eye; LG, lamina ganglionalis; ME, medulla externa; MI, medulla interna. Scale bars: 0.5 mm (A, B), 50 μm (C–G).

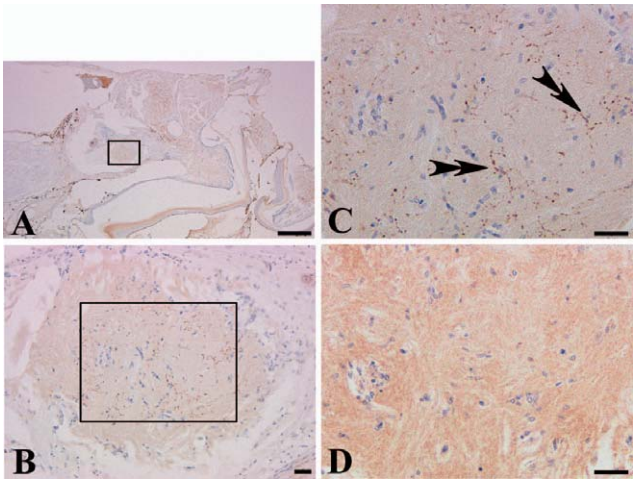

Fig. 6. **(A)** Sagittal section through the thoracic ganglion incubated with aCII6. The rostral side is to the left. **(B)** The boxed area in (A) at a higher magnification. **(C)** The boxed area in (B) at a higher magnification. The double arrowheads indicate GnRH-like-ir fibers. **(D)** Section adjacent to (C) incubated with aCII6 preabsorbed with cGnRH-II. Scale bars: 0.5 mm (A), 50 μm (B–D).

excess amount of cGnRH-II (Fig. 6D). In the abdominal ganglion and the ovary, no GnRH-like-ir cell bodies or fibers were detected (data not shown).

DISCUSSION

The displacement curve obtained for the serially diluted extracts of the kuruma prawn brain paralleled the cGnRH-II standard curve. The kuruma prawn brain and eyestalk extracts showed a similar retention time to synthetic lGnRH-II and octGnRH in rpHPLC combined with TR-FIA analysis, but did not show a similar retention time to cGnRH-II. The cross-reactivities of aCII6 with other GnRH peptides indicated that two forms of GnRH-like peptide, an lGnRH-II-like peptide and possibly an octGnRH-like peptide, exist in the brain and eyestalk of the kuruma prawn. We previously suggested the presence of at least two GnRH-like peptides in the spear squid (Amano et al., 2008). Furthermore, the least two and three GnRH-like peptides were reported in the black tiger shrimp (Ngernsoungnern P et al., 2008) and the giant freshwater prawn (Ngernsoungnern A et al., 2008b), respectively. These findings suggest that in any species of animal, either vertebrate or invertebrate, there are at least two isoforms of GnRH (Gorbman and Sower, 2003).

GnRH-like-ir cell bodies were detected in the anterior-most part of the brain, the protocerebrum. They were located in close proximity to but more caudal than the medium-sized neurons of neuronal cluster 6, which are located in the anterior-most part of the shrimp brain (Sandeman et al., 1992). GnRH-like-ir fibers were observed in the protocerebrum and deutocerebrum. These results suggest that the GnRH-like peptide produced in the protocerebrum is transported caudally to function as a neuromodulator; the deutocerebrum and tritocerebrum are mainly involved in olfaction and mechanosensory functions, respectively (Sandeman et al., 1992).

In the eyestalk, GnRH-like-ir cell bodies were detected in the medulla interna. A large number of GnRH-like-ir fibers were detected in the medulla interna and lamina ganglionalis, and a few GnRH-like-ir fibers were observed in the medulla externa. Since the X-organ in the eyestalk is the site where a number of neurohormones are synthesized, including molt-inhibiting hormone, gonad-inhibiting hormone, mandibular organ-inhibiting hormone, and crustacean hyperglycemic hormone (Huberman, 2000), the GnRH-like peptides may be involved in the regulation of the synthesis and secretion of neuropeptides in the X-organ-sinus gland complex in the kuruma prawn. On the other hand, no GnRH immunoreactivity was detected in the eyestalks of the black tiger shrimp (Ngernsoungnern P et al., 2008) or giant freshwater prawn (Ngernsoungnern A et al., 2008b). Since aCII6 was not used in these studies, the possibility that GnRH-like peptides exist in the eyestalks of the black tiger shrimp and the giant freshwater prawn cannot be ruled out.

In crustaceans, putative gonad stimulating hormone (GSH) is present in the thoracic ganglion and is released by serotonin (Huberman, 2000; Meeratana et al., 2006). In the present study, GnRH-like-ir fibers were detected in the thoracic ganglion. Thus, it may be possible that GnRH-like peptides are involved in GSH secretion in the kuruma prawn, as is also suggested in the giant freshwater prawn (Ngernsoungnern A et al., 2008b), although the relationship between GnRH and GSH needs further clarification.

Although GnRH-like-ir cell bodies and fibers were detected in the CNS of the kuruma prawn, no GnRH-like immunoreactivity was detected in the ovary. Thus, whether a GnRH-like-ir peptide in the CNS directly regulates gonadal function in the kuruma prawn is unclear. In the black tiger shrimp and giant freshwater prawn, lGnRH-I-like immunoreactivity was detected in the ovary (Ngernsoungnern A et al., 2008a, b; Ngernsoungnern P et al., 2008). Furthermore, the administration of mGnRH, sGnRH, and lGnRH-I exerted a strong stimulatory effect on ovarian maturation in the black tiger shrimp (Ngernsoungnern A et al., 2008a). However, it should be noted that no lGnRH-I-like-ir cell bodies or fibers were detected in the CNS of the black tiger shrimp (Ngernsoungnern A et al., 2008a; Ngernsoungnern P et al., 2008) or in that of the giant freshwater prawn (Ngernsoungnern A et al., 2008b). Thus, at present, the effect of endogenous GnRH in the CNS on ovarian maturation in crustaceans is unclear.

In summary, we detected and localized GnRH-like-ir peptide(s) in the CNS of the kuruma prawn by rpHPLC combined with TR-FIA analysis and by immunohistochemistry. GnRH-like peptides chromatographically and immunologically similar to lGnRH-II and octGnRH were detected in the brain and eyestalk. Furthermore, GnRH-like-ir cell bodies were detected in the anterior-most part of the brain and the eyestalk, and GnRH-like-ir fibers were observed in the brain, eyestalk, and thoracic ganglion. The physiological function of the peptide should be examined in a subsequent study, and it is also necessary to clone crustacean GnRH in the near future.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for Scientific Research (B) (No. 20380115) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to M. A. We thank Dr. Koichi Okuzawa of the Ishigaki Tropical Station, Seikai National Fisheries Research Institute, Fisheries Research Agency, Japan, for donating aCII6. We also thank Mr. Yoshinori Nagai, Ms. Mio Matsuki, Mr. Susumu Shimogaki, and Ms. Mineka Hotta of the School of Marine Biosciences, Kitasato University, for their help in this study.

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(Received July 21, 2009 / Accepted August 25, 2009)