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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 fluorimunoassay (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 protostomes Chelyosoma productum (Powell et al., 1996) and Ciona intestinalis (Adams et al., 2003), and the cephalopod Octopus vulgaris (Iwakoshi et al., 2002). Moreover, the full-length cDNA of a GnRH-like mole-

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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 japonicus), which is the most commercially important species in shrimp aquaculture in Japan, by reverse-phase high performance liquid chromatography (rpHPLC) combined with time-resolved fluorimunoassay (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 tunicate GnRH-I (tGnRH-I), IgGnRH-II, lGnRH-II, and catfish GnRH. In this study, we further examined the cross-reactivities of aCII6 against salmon GnRH (sGnRH), seabream GnRH, IgGnRH-I, mammalian GnRH (mGnRH), cGnRH-I, and catfish GnRH. In this study, we further examined the cross-reactivities of aCII6 against tunicate GnRH-I (IgGnRH-I), IgGnRH-II, IgGnRH-III, and IgGnRH-II, dogfish GnRH, herring GnRH, medaka GnRH (pejerrey GnRH), whitefish GnRH, frog GnRH, guinea pig GnRH, and octGnRH. The cross-reactivities of aCII6 against IgGnRH-II (41.8%) and octGnRH (47.8%) were relatively high, as summarized in Table 1.

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

<table>
<thead>
<tr>
<th>GnRH forms</th>
<th>Cross-reactivity (%)</th>
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<tbody>
<tr>
<td></td>
<td>anti-cGnRH-II (aCII6)</td>
</tr>
<tr>
<td>Mammalian GnRH</td>
<td>(mGnRH)</td>
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<tr>
<td>Guinea pig GnRH</td>
<td>(gpGnRH)</td>
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<tr>
<td>Chicken GnRH-I</td>
<td>(cGnRH-I)</td>
</tr>
<tr>
<td>Chicken GnRH-II</td>
<td>(cGnRH-II)</td>
</tr>
<tr>
<td>Frog GnRH</td>
<td>(frGnRH)</td>
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<tr>
<td>Salmon GnRH</td>
<td>(sGnRH)</td>
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<tr>
<td>Catfish GnRH</td>
<td>(cfGnRH)</td>
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<tr>
<td>Seabream GnRH</td>
<td>(sbGnRH)</td>
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<tr>
<td>Medaka GnRH</td>
<td>(mdGnRH)</td>
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<tr>
<td>Herring GnRH</td>
<td>(hrGnRH)</td>
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<tr>
<td>Dogfish GnRH</td>
<td>(dfGnRH)</td>
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<tr>
<td>Whitefish GnRH</td>
<td>(wfGnRH)</td>
</tr>
<tr>
<td>Lamprey GnRH-I</td>
<td>(lGnRH-I)</td>
</tr>
<tr>
<td>Lamprey GnRH-II</td>
<td>(lGnRH-II)</td>
</tr>
<tr>
<td>Lamprey GnRH-III</td>
<td>(lGnRH-III)</td>
</tr>
<tr>
<td>Tunicate GnRH-I</td>
<td>(tGnRH-I)</td>
</tr>
<tr>
<td>Tunicate GnRH-II</td>
<td>(tGnRH-II)</td>
</tr>
<tr>
<td>Octopus GnRH</td>
<td>(octGnRH)</td>
</tr>
</tbody>
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RESULTS

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 (Monoset, 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, IgGnRH-II or octGnRH (10 μg cGnRH-II, IgGnRH-II, or octGnRH in 1 ml antisera). 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).

TR-FIA

The displacement curve obtained for the serially diluted extracts of the kuruma prawn brain paralleled the cGnRH-II standard curve (Fig. 1).
The kuruma prawn brain and eyestalk extracts showed a similar retention time to 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 CH₃CN (acetonitrile) containing 16 mM TEAA. The dotted lines represent the percentage of acetonitrile in the mobile phase.

**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 CH₃CN (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. AINV, 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).
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 ganglonalis (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 excess amount of cGnRH-II (Fig. 6G), lGnRH-II, and octGnRH (data not shown).
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 ganglonalisa, 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.

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