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Immunocytochemical localization of neuropeptide Y, serotonin, substance P and β-endorphin in optic ganglia and brain of *Metapenaeus ensis*[*]

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Abstracts By using immunocytochemistry method of Strept Avidin-Biotin-Complex, four kinds of antisera raised against rabbits were applied to observe the immunoreactive neurons and neuropils of serotonin (5-HT), neuropeptide Y (NPY), substance P (SP) and β-Endorphin (β-Ep) in optic ganglia and brain of *Metapenaeus ensis*. The results showed that, the 5-HT-immunoreactive cells were located in all the four neuropils of optic ganglia. Immunoreactivity of 5-HT was detected in anterior medial protocerebrum neuropils (AMPN), and the inner and outer lateral beside olfactory lobe (OL) of deutocerebrum. The presence of NPY-immunoreactive cells was found in all the four neuropils of the optic ganglia. NPY-immunoreactivity occurred in the anterior median cell cluster, lateral cell cluster of protocerebrum, and cell cluster beside OL and AMPN. SP-immunoreactivity was found in medulla terminalis (MT) of optic ganglia, and lateral cell cluster of protocerebrum and posterior lateral cell cluster of tritocerebrum. β-Ep-immunoreactive cells were in MT only. In conclusion, these specific distribution patterns of the four immunoreactive substances can be used as morphological clues for understanding their different neurophysiological functions.

Key words: *Metapenaeus ensis*; optic ganglia; brain; immunocytochemistry

1 INTRODUCTION

Amines and peptides are important bioactive substance in crustaceans, and they function as hormones, neurotransmitters and neuromodulators. By far, bioactive substances that have been detected from nervous system of crustaceans include serotonin (5-HT), dopamine, octopamine, Leu-enkephalin, β-Endorphin (β-Ep), substance P (SP), neuropeptide Y (NPY), Glucagon, vasopressin, melanocyte stimulating hormone, oxytocin, vasotocin, somatostation, gastrin, and calcitonin, etc (Fingerman et al., 1993, 1994; Harzsch and Glőtzner, 2002; Ye et al., 2004). *Metapenaeus ensis*, belonging to *Metapenaeus*, Penaeidae, Natantia, Decapoda, Crustacea, is a kind of commercially important shrimps. So far, studies on *M. ensis* have mainly focused on its aquaculture biology, but none on neuroendocrinolgy. In the present study, antibodies of 5-HT, NPY, SP and β-Ep, and immuocytological techniques of Strept Avidin-Biotin-Complex (SABC), were applied to distinguish and locate the four kinds of neurons in optic ganglion and brain of *M. ensis*. Particular distribution patterns of these immunoreactive substances may be used as morphological clues for understanding their neuroendocrine activities.

2 MATERIALS AND METHODS

2.1 Tissue preparation

Adult shrimps (*M. ensis*; body length of 11.4–13.8 cm) were obtained in local market in

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Xiamen South China. A total of 18 shrimps were used in this study. The brain and the optical ganglia were dissected separately, completely desheathed and fixed in Bouin's solution for 16–18h at 4℃. The tissues were then embedded in paraffin after routine dehydration in alcohol and clearing in xylene. Serial sagittal and cross sections in 8 μm were put on clear glass slides.

2.2 Main reagent

Primary antisera generated in rabbits against 5-HT, β-Ep and SP (1:50 dilution) were produced by ZYMED Company. Primary antisera generated in rabbits against NPY (ready-to-use) and 3', 3'-diaminobenzidine (DAB) were produced by Sigma Company. The SABC kit was purchased from Wuhan Boster Biological Technology LTD.

2.3 SABC Immunocytochemistry method

To detect 5-HT, β-Ep, SP and NPY, the sections were immunocytochemically stained in SABC method. The following procedure was performed. Tissues were (1) incubated in 3% H_2O_2/PBS for 10 min to inactivate endogenous peroxidase at room temperature; (2) incubated in normal goat serum (1:10) for 10min to reduce non-specific binding at room temperature; (3) incubated in primary antisera for 1.5 h at 37℃; (4) incubated in Biotinylated goat anti-rabbit IgG for 0.5 h at 37℃; (5) incubated in SABC for 0.5 h at 37℃; (6) after 5–10 min in 0.06%DAB-0.03%H_2O_2, sections were dyed in hematoxylin, dehydrated in alcohol, cleared in xylene and cover slipped; (7) preparations were viewed and photographed with an Olympus BX51 microscope. Control sections were prepared simultaneously by substituting normal goat serum (1:10) in place of the primary antibodies.

3 RESULT

The brain of *M. ensis* is composed of protocerebrum, deutocerebrum and tritocerebrum. Neuropils in optic ganglia can be divided into four parts, lamina ganglionaris (LG), medulla externa (ME), medulla interna (MI) and medulla terminalis (MT). Most immuoreactive cells distinguished themselves by their brown plasma with brown immunoreactive granules inside. Nevertheless, the nuclears of the immuoreactive cells did not have any immunoreactivity and thus took the shape of vacuoles. Control sections showed negative reaction.

3.1 The optic ganglia

Small 5-HT- immunoreactive (5-HT-IR) neurons distributed sporadically among MT, MI, and outer side of ME. There are some large 5-HT-IR neurons in MT, with several ones in MTXO (Fig.1:1).

NPY-immunoreactive (NPY-IR) neurons distributed in inner margin of LG (Fig.1:2), outer margin of ME, inner margin of ME adjacent to MI and MT, and inner margin of MI (Fig.1:3). NPY-IR neurons in MT are large and densely stained, and they located in outer margin, central part and the side near the optic nerve (Fig.1: 4 and 5). MT has the largest number of NPY-IR neurons. Neuropils except LG showed immuoreactivity, and in MT, they gathered near MTXO.

Being lightly immuno-labeled, SP-immunoreactivity (SP-IR) neurons were seen only on the outer margin of MT (Fig.1:6), and immuoreativity was not found in other parts of optic ganglia.

β-Ep-immunoreactivity (β-Ep-IR) neurons, with weak immunoreactivity, existed in MT adjacent to MI, the central MT, and the MTXO (Fig.1:7).

Sinus gland showed no immunoreactivity against the above-mentioned antisera.

3.2 The brain

5-HT-IR neurons located in the anterior cell cluster (Fig.1:8) and the outer cluster beside OL of deutocerebrum. The former have less 5-HT-IR neurons than the latter. No 5-HT-immunoreactivity was found in neuropils in the brian.

NPY-IR neurons distributed in anterior median cluster and lateral cluster of protocerebrum, inner anterior cluster and outer cluster beside olfactory lobe (OL). There were more NPY-IR neurons in anterior median cell cluster and the inner cell cluster beside OL (Fig.1:9, 10). Fewer NPY-IR neurons were in outer cell cluster beside OL (Fig.1:11). The anterior medial protocerebrum neuropils (AMPN) showed strong immunoreactivity (Fig.1:9), while OL showed weaker immunoreactivity. Other neuropils did show no immunoreactivity.

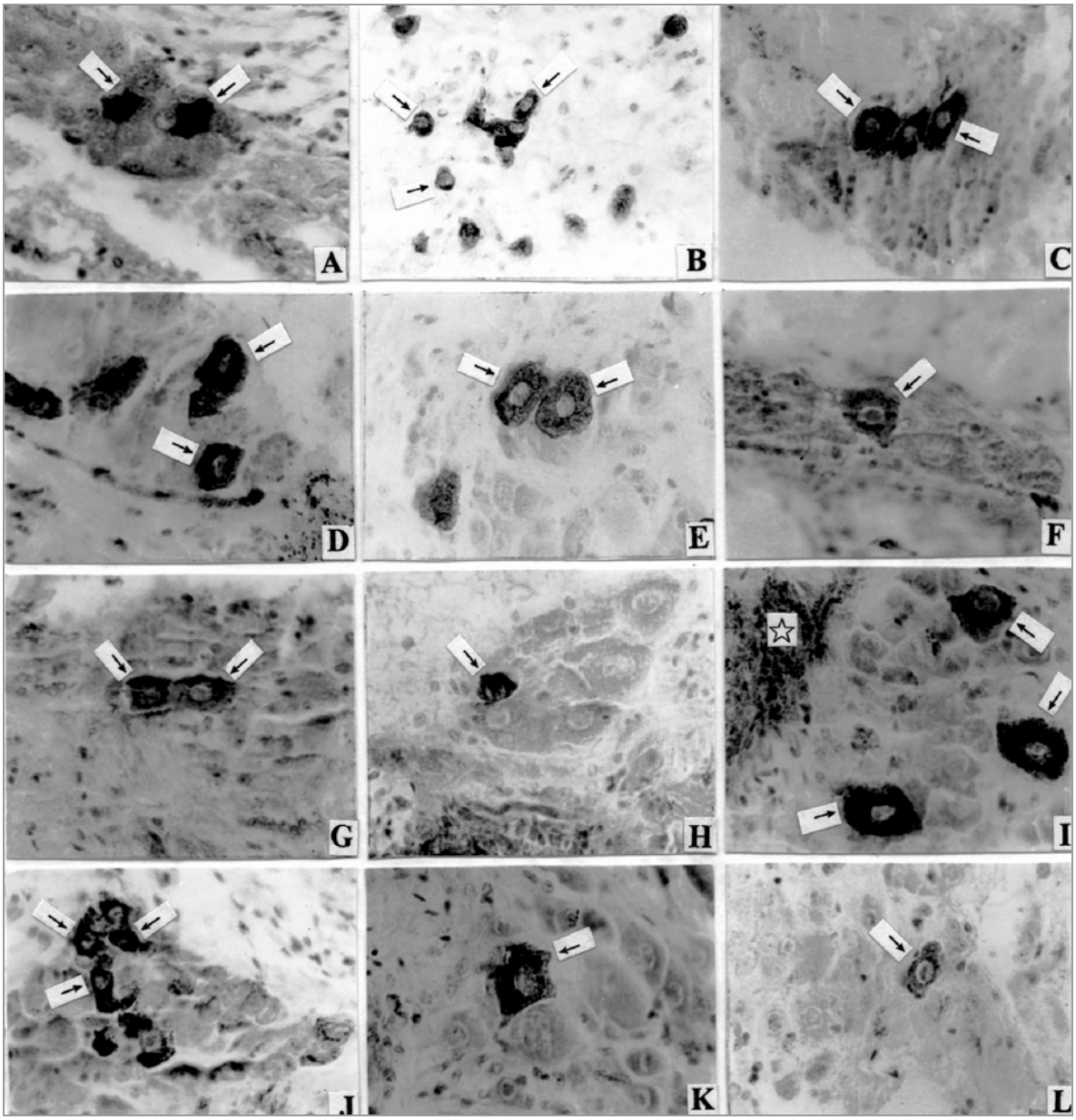

Fig.1 Immunoreactivity of optic ganglia and brain of *Metapenaeus ensis*

1. 5-HT-IR neurons (arrows; same hereafter, and specify otherwise) in MTXO; 2. NPY-IR neurons in inner margin of LG; 3. NPY-IR neurons in inner margin of MI; 4. NPY-IR neurons in outer margin of MT; 5. NPY-IR neurons in central part of MT; 6. SP-IR neurons in outer margin of MT; 7. β-Ep –IR neurons in MT adjacent to MI; 8. 5-HT-IR neurons in cell cluster beside OL; 9. NPY-IR neurons in anterior medial cell cluster of protocerebrum and immunoreactive AMPN (indicated by the star); 10. NPY-IR neurons in inner cell cluster beside OL; 11. NPY-IR neurons in outer cell cluster beside OL; 12. SP-IR neurons in lateral cell cluster of protocerebrum ; ×330

Being weakly immunostained, SP-IR neurons were found in lateral cell cluster of protocerebrum and posterior lateral cell cluster of tritocerebrum (Fig.1:12).

No β-Ep-immunoreactivity was detected in the brain.

4 DISCUSSION

4.1 5-HT immunoreactivity

5-HT is one of the important bioamines. Applied with immunocytological, radioimmunological

techniques, HPLC and other techniques, 5-HT has been detected for location in optic ganglion, brain, thoracic ganglion, stomatogastric nervous system and pericardial organ of crustaceans (Fingerman et al., 1993). Wide distribution of 5-HT in central and peripheral nervous system implies that it may have various physiological functions.

Distribution of 5-HT-IR cells in optic ganglion of crustaceans has often been reported. In *Cancer antennarius*, there are many 5-HT- IR cells in outer margins of ME and MI, while there is only one separate cell below sinus gland and in the X-organ (Rudolph and Spaziani, 1990). In *Pacifastacus leniusculus*, similarly, there are some immuoreactive cells in outer edges of ME and MI, but the immunoreactivity of neuropils is stronger, forming three immunoreactive bands separately in ME and MI. Immunoreative cells in MT mainly are located at outer margin of abdominal side, and some immunoreactive nerve fibers in optic ganglia enter the protocerebrum (Elofsson, 1983). Recently, 5-HT-IR neurons were detected in all the four neuropils of optic ganglia in *Litopenaeus vannamei* (Ye et al., 2004). 5-HT-IR neurons in optic ganglia of *M. ensis* were found in three neuropils other than in LG, while no immunoreactivity has been detected in any neuropils. This result is quite different from aforementioned species, but similar to that of *Palaemon serratus* (Bellon-Humbert and van Herp, 1988). Studies indicated that 5-HT can adjust many neurohormones in optic ganglion. For example, 5-HT can modulate the secretion of red pigment dispersing hormone, induce proximal retinal pigment in retinal cells to transfer entirely to dark places, enhance the combination and secretion of MIH in the X-organ of *in vitro* eyestalk, and activate the release of crustacean hyperglycemic hormone (CHH) in sinus gland (Fingerman, 1993). Wide distribution of 5-HT- Immunuoreactivity in MT, MI, and ME, except in sinus gland (neurohemic organ) in optic ganglion of *M. ensis* implies that 5-HT may play role as a neurotransmitter and engage in synthesis and secretion of related neurohormones.

In *Pacifastacus leniusculus*, 5-HT-IR neurons distribute among anterior median cell cluster, posterior median cell cluster, and inner cell cluster beside OL. 5-HT immunoreactivity was also found in neuropils as protocerebral bridge, central body and OL, etc (Elofsson, 1983). In *Litopenaeus vannamei*, 5-HT immunoreactivity was detected in anterior median cell cluster, AMPN, inner and outer cell clusters beside OL (Ye et al., 2004). In *M. ensis*, 5-HT-IR neurons distribute in anterior cell cluster and outer cluster beside OL. Johansson (1991) divided the intermediate olfactory neurons in inner cell cluster beside OL into 2 types: large and small 5-HT-IR cells. *Macrobrachium rosenbergii* have only large 5-HT-IR cells, and *Munida sarni* have only small ones, while both types have been detected in *Pacifastacus leniusculus* and *Hyas araneus*. Therefore, the diversity of intermediate olfactory neurons may imply the difference in processing olfactory information. Only one type of 5-HT-IR neurons, which was named as the Type III neurons in historical study, has been found in anterior cell cluster beside OL of *M. ensis*. Reportedly, 5-HT could stimulate the brain and thoracic ganglia of *Procambarus clarkii* and *Uca pugilater* to secrete gonad stimulating hormone (GSH), thus accelerate the gonadal development (Fingerman, 1997). Our study provides the morphological clues of possible function of 5-HT in stimulating the secretion of GSH in *M. ensis* 's brain.

4.2 NPY immunoreactivity

NPY belongs to the Pancreatic Polypeptide, for modulating feeding, sexual activity, blood pressure and physiological rhythm of vertebrates (Hoyle, 1998). Researches on NPY distribution in crustaceans are quite limited. Only a few NPY-IR neurons in ME and MI of *Homarus gammarus*, and the sinus gland were showed with immunoreactivity (Charmantier-Daures, 1987). The immunoreactivity of NPY was found in all the four neuropils of optic ganglia, anterior medial cell cluster and AMPN of brain in *Litopenaeus vannamei* (Ye et al., 2004). Likewise, NPY-IR neurons were detected in all the four neuropils in optic ganglia of *M. ensis*. However, NPY-immunoreactivity appears in neuropils of protocerebrum, OL, anterior medial cell cluster, anterior lateral cell cluster, and inner and outer cell cluster beside OL in its brain, similar to that in fresh water crab *Chiromantes haematocheir* (Yoshiharu et al., 1996). Wide distribution of NPY immunoreactivity in protocerebrum and OL of *M. ensis* indicated the NPY involvement in physiological activities, such as olfactory formation and feeding.

So far, NPY has been found in protozoan, coelenterates, crustaceans and vertebrates (Zhang et al., 1993; Gu et al., 1998; Ye et al., 2004). It is concluded that NPY has certain conservatism in biological evolution in terms of its distribution in different phyla.

4.3 SP immunoactivity

SP widely distributes in nervous system and peripheral organs of vertebrates, and play roles in exciting the muscle fiber, stretching the blood vessels, stimulating the secretion of glands, exciting motor neurons in spinal cord, engaging in axonal reflexes, modulating body fluid balance, and receiving and transferring pain signals (Gu, 1985). By Applying SP antiserum raised against vertebrates, the immunoreactivity was found in the four neuropils and sinus gland in optic ganglia of *Pacifastacus interruptus*, and in the retina cell and sinus gland of *Uca pugilator* (Mancillas et al., 1981; Fingerman et al., 1985). The existence of SP in sinus gland indicates that SP functions as neurohormone. In the optic ganglia of *M. ensis*, only MT has immunoreaction. There were two large separate SP-IR neurons in protocerebrum and deutocerebrum of *Leptograpsus variegates* and *Cherax destructor*, extending their cellular projections into OL (Langworthy et al., 1997). SP-immunoreactivity distributes widely in the brain of *Hamarus americanus*, except the neuropils in OL of deutocerebrum, median antenna I neuropil and lateral antenna I neuropils (Sandeman et al., 1990). SP-IR cells existed in anterior median cell cluster of protocerebrum of *Litopenaeus vannamei* (Ye et al., 2004). However, in *M. ensis*, SP-immunoreactivity was detected only in anterior lateral cell cluster and posterior lateral cell cluster. It remains unsolved whether the SP immunoreactivity difference in the nervous system of different crustaceans is caused by species difference.

4.4 β-Ep immunoreactivity

β-Ep is mostly secreted by hypothalamic arcuate nucleus and medial lobe of pituitary body. In addition to easing pain and maintaining relative consistence of neuroendocritic activities, β-Ep can act on lymphomas via corresponding receptors, thus widely influence the immune system (Cao et al., 1999). However, little has been known about the distribution and function of β-Ep in nervous system of crustaceans. Ye et al. (2004) reported, β-Ep-IR cells existed in posterior lateral cell cluster of tritocerebrum of *Litopenaeus vannamei*, while no immunoreactivity was detected in its optic ganglia. These results were quite different from those in *M. ensis*. MTXO in optic ganglia of crustaceans is a synthesis center for neuropeptides of CHH family. There are some β-Ep-IR neurons inside and near MTXO of *M. ensis*. Probably these neurons participate in modulating the synthesis of CHH family neuropeptides. However, no β-Ep-immunoreactivity was detected in its brain. Further investigation on distribution and function of β-Ep in nervous system of crustaceans are suggested.

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