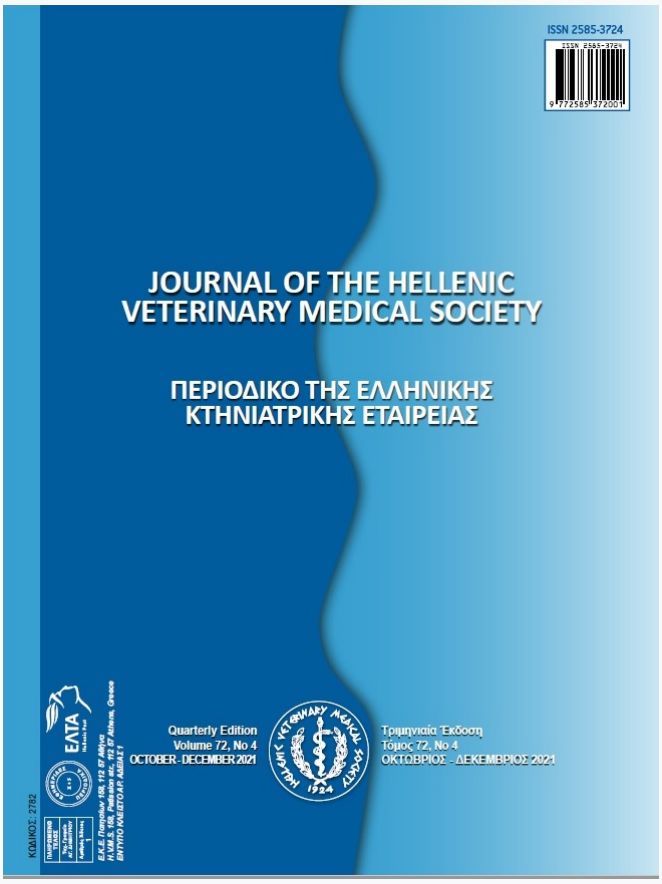
Journal of the Hellenic Veterinary Medical Society

Vol 72, No 4 (2021)



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doi: 10.12681/jhvms.29371

To cite this article:

SHA, A., & HAO, H. (2022). Expressions and Distributions of Leucine-enkephalin, Delta, Mu, and Kappa Opioid Receptors in Four Systems of the Octopus, Octopus ocellatus by Comparative Immunohistochemical Method and ELISA. *Journal of the Hellenic Veterinary Medical Society*, *72*(4), 3337–3344. https://doi.org/10.12681/jhvms.29371

J HELLENIC VET MED SOC 2021, 72(4): 3337-3344
ΠΕΚΕ 2021, 72(4): 3337-3344

Research article
Ερευνητικό άρθρο

Expressions and Distributions of Leucine-enkephalin, Delta, Mu, and Kappa Opioid Receptors in Four Systems of the Octopus, *Octopus ocellatus* by Comparative Immunohistochemical Method and ELISA

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ABSTRACT: We have investigated the expressions and distributions of leucine-enkephalin (Leu-enk), delta (δ), mu (μ), and kappa (κ) opioid receptors immunoreactivities in the respiratory, circulatory, excretory and reproductive systems of the Octopus, *Octopus ocellatus*, by comparative immunohistochemical method and enzyme-linked immunosorbent assay (ELISA). The results showed that δ opioid receptor and Leu-enk immunoreactivities were both detected in the branchia, ventricle, branchial heart, ovary and oviducal gland, while δ opioid receptor was additionally detected in the branchial gland and kidneys in less extend. The majority of the examined tissues presented weak immunoreactivities of δ opioid receptor and Leu-enk, with the notable exception of the white body and spermary tissues that were found negative. No labellings of μ and κ opioid receptors were observed in the respiratory, circulatory, excretory and reproductive systems of *O. ocellatus*. The combined results of δ opioid receptor and Leu-enk immunoreactivities indicated that they may be involved in the regulations of respiration, circulation, reproduction and endocrine together with other hormones or neurotransmitters in the *O. ocellatus*' body. The different densities of δ opioid receptor and Leu-enk in the respiratory, circulatory, excretory and reproductive systems of *O. ocellatus* may be related to the different functions.

Keywords: *O. ocellatus*, leucine-enkephalin, opioid receptors, respiratory system, circulatory system, excretory system, reproductive system

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Date of initial submission: 27-07-2020
Date of acceptance: 16-05-2021

INTRODUCTION

Among the family of endogenous opioid peptides, Enkephalin (Enk) is considered to play an important role in the regulations of cardiovascular, digestive, endocrine, and immune systems of animals (Eccles 1990; Makman 1994). Enk mainly includes leucine-enkephalin (Leu-enk), methionine-enkephalin (Met-enk), met-enkephalin-Arg^6-Gly^7-Leu^8, met-enkephalin-Arg^6-Phe^7, and is widely distributed in the nervous system of higher animals. So far, three kinds of opioid receptors have been identified in mollusk and higher animals, namely delta (δ), mu (μ) and kappa (κ) opioid receptors. Delta opioid receptor is involved in the analgesic effect of the endogenous opioid peptides of spinal cord, and participates in the regulations of emotion, incretion and cardioprotective effect through ischemic preconditioning. Because of the strong interaction with δ opioid receptor, Enk is considered to be the endogenous ligand of the receptor (Stefano et al. 1993a; Sha et al. 2012).

The opioid receptors and Enk have been found in mollusk. First reports originated from Stefano and Catapane (1979), who investigated the regulation of dopamine levels in the central nervous system (CNS) of *Mytilus edulis* by exogenous opioids. Then, Leung and Stefano (1984) isolated and sequenced Leu-enk and Met-enk from the pedal ganglia of *M. edulis*, and sequential amino acid analysis showed that these peptides share the same primary structure as that of vertebrates. The above-mentioned authors purified a heptapeptide from these fractions of invertebrates by high-pressure liquid chromatography under isocratic conditions. Sequential amino acid analysis demonstrated that this heptapeptide share the same primary structure as the met-enkephalin-Arg^6-Phe^7 of invertebrates. In addition, Stefano et al. (1989, 1996) have shown that there were two kinds of δ opioid receptors in the immunocytes and the nervous tissue of *M. edulis*, those are δ-1 and δ-2. Moreover, the participation of δ, μ and κ opioid receptors has been demonstrated in the digestive regulation of *Limax maximus* (Kavaliers et al. 1986), as well as in pain modulation of *Cepaea nemoralis* (Thomas 1997). More recently, Cadet (2004) have detected the μ transcripts from the pedal ganglia of *M. edulis*. These results indicate that invertebrates such as *M. edulis* possess enkephalinergic systems similar to those found in higher organisms.

In our previous study, we identified the presence of δ opioid receptor and Leu-enk in mantles and feet of *O. ocellatus*, using immunohistochemical techniques (Sha et al. 2012). The aim of the present study was to investigate the expressions and distributions of Leu-enk, δ, μ, and κ opioid receptors immunoreactivities in the respiratory, circulatory, excretory and reproductive systems of the Octopus, *Octopus ocellatus*. The results will provide baseline information for future investigation regarding the roles of Leu-enk and opioid receptors of δ, μ, and κ in the respiratory, circulatory, excretory and reproductive systems of adult mollusk.

MATERIALS AND METHODS

All mentioned procedures were carried out in agreement with Chinese legislation on experimental animals, after approval by the Ethic-Scientific Committee for Experiments on Animals of Chongqing Three Gorges University.

Experimental Animals

A batch of adult male and female Octopuses, *O. ocellatus* (n = 10 for each sex), ranging from 15 to 21 cm in length, were purchased from a commercial farm in Yantai (China). They were kept for 3 days in large tanks with aerated circulating seawater maintained at 20-21°C and fed ad libitum with living crabs and shrimps.

Preparation of the Respiratory, Circulatory, Excretory and Reproductive Systems Tissues in Immunohistochemistry

The tissues of branchia, branchial gland, ventricle, white body, branchial heart, kidneys, ovary, oviducal gland and spermary were dissected and fixed for 5-7 h in a solution of 4% paraformaldehyde (the chemicals of preparation part were purchased from Sinopharm Chemical Reagent Co., Ltd., China). The tissues were washed three times in 0.05 M phosphate-buffered saline (PBS, pH 7.4, 2% sodium chloride) every 30 minutes. Cryostat sections (20 μm thick) of the tissues were placed on chromalum-gelatin-coated glass slides and rehydrated in 0.05 M PBS containing 0.3% Triton X-100 (PBST, pH 7.4). After dried, the frozen sections were stored in the refrigerator at 4°C.

Immunohistochemistry

The immunohistochemical SABC reaction program was carried out according to our previous studies (Sha et al. 2012, 2013). The sections were incubated with rabbit anti-mouse Leu-enk, δ, μ, or κ opioid receptors (Sigma Chemical Co., USA, diluted 1:400

in blocking buffer), washed with PBST (Sinopharm Chemical Reagent Co., Ltd., China), then incubated with goat anti-rabbit IgG (Santa cruz Biotechnology, Inc., CA, USA, diluted 1:200) and streptavidin biotin peroxidase complex protein (AB composite liquid, Santa Cruz Biotechnology, Inc., CA, USA) in turn. Finally, the reaction was visualized using the diaminobenzidine (DAB, Fluka Chemie AG, Switzerland), and the nuclei were counterstained with hematoxylin (Sigma Chemical Co., USA).

Imaging

The slides were observed under a BX50 Light Microscope (Olympus Corporation, Japan) and digital images were obtained using a DP70 Camera System (Olympus Corporation, Japan) and processed by Adobe Photoshop CS (Adobe Systems Inc., CA, USA). The immunoreactivities of Leu-enk, δ, μ, and κ opioid receptors were based on the appearance of brownish-red staining corresponding to the presence of DAB deposition in the tissues. The nuclei was blue, the cytoplasm and background were not colored or yellowish.

Preparation of the Branchia, Ventricle, Branchial Heart and Ovary Tissues in Enzyme Linked Immunosorbent Assay (ELISA)

The tissues of branchia, ventricle, branchial heart and ovary were collected postmortem *O. ocellatus* and washed with precooled PBS (0.01M, pH = 7.4), in order to remove the residual blood, and final tissues were harvested after weight measuring. The collected tissues were sliced and placed in 9-fold PBS. After adding protease inhibitor (Sigma Chemical Co., USA), 10% tissue homogenate was prepared with ice water bath homogenate for 10min. Finally, the homogenate was centrifuged at 5000 rpm for 8 min, and the supernatant was collected for detection.

ELISA

ELISA was performed according to the Leu-enk ELISA kit instruction provided by Shanghai Jingkang Bioengineering Co., Ltd, Shanghai, China. Stop solution was added to each well for 15 min and the OD value was measured at 450nm using an iMark Microplate Reader (Bio-Rad, USA). Then, the OD value of the sample was substituted into the linear regression equation, and the Leu-enk concentration of the sample was calculated.

RESULTS

Immunohistochemistry

Distributions of Mu and Kappa Opioid Receptors Immunoreactivities in the Respiratory, Circulatory, Excretory and Reproductive Systems

No labellings of μ and κ opioid receptors were observed in the respiratory, circulatory, excretory and reproductive systems of *O. ocellatus*.

Distributions of Leu-enk and Delta Opioid Receptor Immunoreactivities in the Respiratory System

Branchia

The branchia lies in the anterior part of the mantle, composed of a pair of feathery gills. The branchia comprises of the mantle cavity and three types of tissues: a single layer of respiratory epithelium lying atop a dense irregular connective tissue layer and the adjacent inner layer consists of muscle tissue. The results indicated that individual immunopositive cells of Leu-enk and δ opioid receptor were revealed in the respiratory epithelium and connective tissue of the branchia (Fig. 1-A, 2-A, 2-B).

Branchial Gland

The vascular branchial gland is a pair of adjacent glands of the branchia, which extends along the entire dorsal gill's length, without presenting ducts and being quite separate from the tissue of the gills. And each, suspended from the overlying mantle and closely attached to the dorsal surface of the gills, is contained in a capsule consisting of an external epithelial layer of columnar cells upon connective tissue which is well vascularized, and contains longitudinal and oblique muscle fibres. There was no Leu-enk immunoreactivity in the branchial gland (Fig. 1-B). Weak immunoreactivity of δ opioid receptor was detected in the apical part of the columnar epithelial cells and connective tissue of the branchial gland (Fig. 2-C, 2-D).

Distributions of Leu-enk and Delta Opioid Receptor Immunoreactivities in the Circulatory System

Ventricle

The flavescent orbicular-ovate ventricle lies between the two atria, the structure of the ventricle comprises of three layers: a single layer of flattened epithelium lying atop a dense irregular connective tissue layer and the adjacent inner layer consists of myocardium. The results indicated that a small amount of δ opioid receptor and Leu-enk immunoreactive ma-

terial was observed in the epithelium and connective tissues (Fig. 1-C, 2-E). The muscle tissue was found immunonegative for both δ opioid receptor and Leu-enk.

White Body

The white body is a pair of multilobed organs, attached to the medial external surface of each eye and is responsible for hemocyte production by *O. ocellatus*. No labellings of δ opioid receptor and Leu-enk were observed in the white body (Fig. 1-D).

Branchial Heart

The flavescens saccate branchial heart lies at the end of the branchia, the efferent vein leaves the branchial gland to enter the branchial heart, whence the blood passes via the gills to the systemic heart. The structure of the branchial heart comprises of three layers: epithelial tissue, connective tissue and muscle. There were weak stained granules of δ opioid receptor and Leu-enk in the connective tissue of the branchial heart (Fig. 1-E, 2-F). No immunoreactivities were seen in the epithelium and muscle tissues. The epithelial and muscle tissues of the branchial heart were found immunonegative for both δ opioid receptor and Leu-enk.

Distributions of Leu-enk and Delta Opioid Receptor Immunoreactivities in the Excretory System

Kidneys

The renal appendage is a gland-like, highly branched structure protruding in the renal sac. They are formed of continuous sheets covered of two layers of cuboid epithelial cells separated by blood sinuses derived of the vena cava system (Budelmann et al. 1997). No Leu-enk immunopositivy was detected in the kidneys (Fig. 1-F), but weak immunoreactivity of δ opioid receptor was presented in the connective tissue of the kidneys (Fig. 2-G).

Distributions of Leu-enk and Delta Opioid Receptor Immunoreactivities in the Reproductive System

O. ocellatus is dioecious animal. The female reproductive system consists of a single ovary with two oviducts opening on either side of the mantle cavity. The male reproductive system consists of an unpaired testis and a duct opening into the left side of the mantle cavity. The duct is composed of the vas deferens proximal to the testis, followed by the first spermatophoric gland (seminal vesicle), the second spermatophoric gland (prostate), the Needham's sac and the penis.

Ovary

The ovary is located at the posterior part of the visceral mass, which was formed by the epithelial development of the body cavity. The structure of the ovary comprises of two layers: epithelial tissue and connective tissue. The results indicated that weak Leu-enk's immunoreactivity was revealed in the connective tissue of the ovary, and weak immunoreactivity of δ opioid receptor in the epithelial tissue of the ovary (Fig. 1-G, 2-H).

Oviducal Gland

The oviducal gland is the enlarged part at the bottom of the fallopian tube, which is about half the size of the fallopian tube. It is a compact ovoid-shaped gland that exhibits two different sectors, an outer sector and an inner one, and it is covered with a thin connective-muscular capsule. The gland appears organized in compact lobes of branched glandular epithelial tubules, separated by thin connective septa. Diffuse Leu-enk immunoreactivity was scattered in the gland ciliary cells of central gland (Fig. 1-H) and general or weak immunoreactivity of δ opioid receptor was detected in the gland cells of peripheral gland's epithelial tissue of the oviducal gland (Fig. 2-I), the other examined tissues were found immunonegative.

Spermary

The spermary lies in back of the visceral mass, which was also formed by the epithelial development of the body cavity. The spermary was found immunonegative for both δ opioid receptor and Leu-enk (Fig. 1-I).

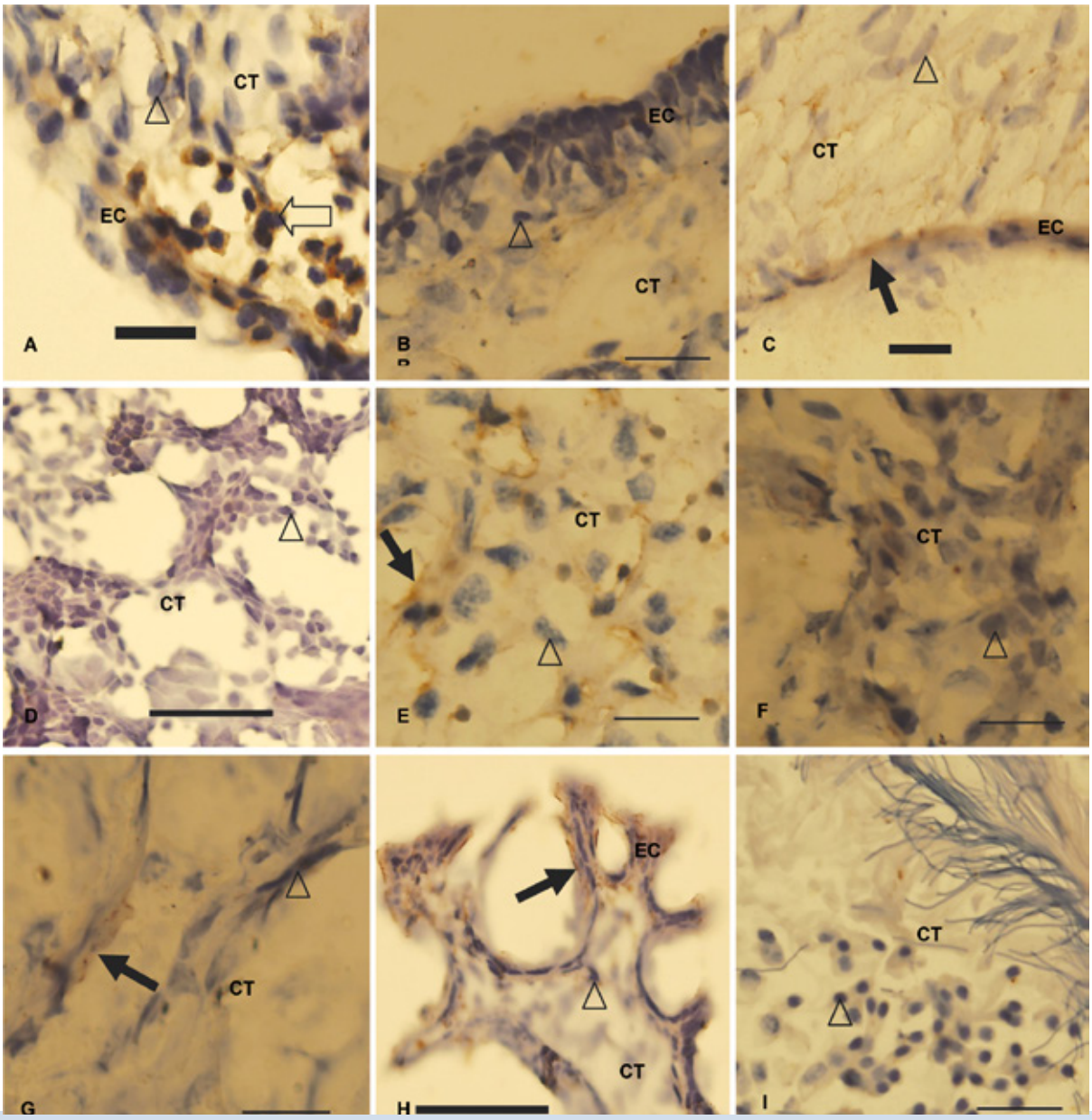

Fig.1 Distribution of Leu-enk Immunoreactivity in the respiratory, circulatory, excretory and reproductive systems

A. Distribution of Leu-enk Immunoreactivity in the branchia; B. Distribution of Leu-enk Immunoreactivity in the branchial gland; C. Distribution of Leu-enk Immunoreactivity in the ventricle; D. Distribution of Leu-enk Immunoreactivity in the white body; E. Distribution of Leu-enk Immunoreactivity in the branchial heart; F. Distribution of Leu-enk Immunoreactivity in the kidneys; G. Distribution of Leu-enk Immunoreactivity in the ovary; H. Distribution of Leu-enk Immunoreactivity in the oviducal gland; I. Distribution of Leu-enk Immunoreactivity in the spermary. Scale bars in D and H=50μm, the others=20μm.
Black arrows-positive Leu-enk granules; white arrows-positive cells; open triangles-karyon; EC-epithelium; CT-connective tissue.

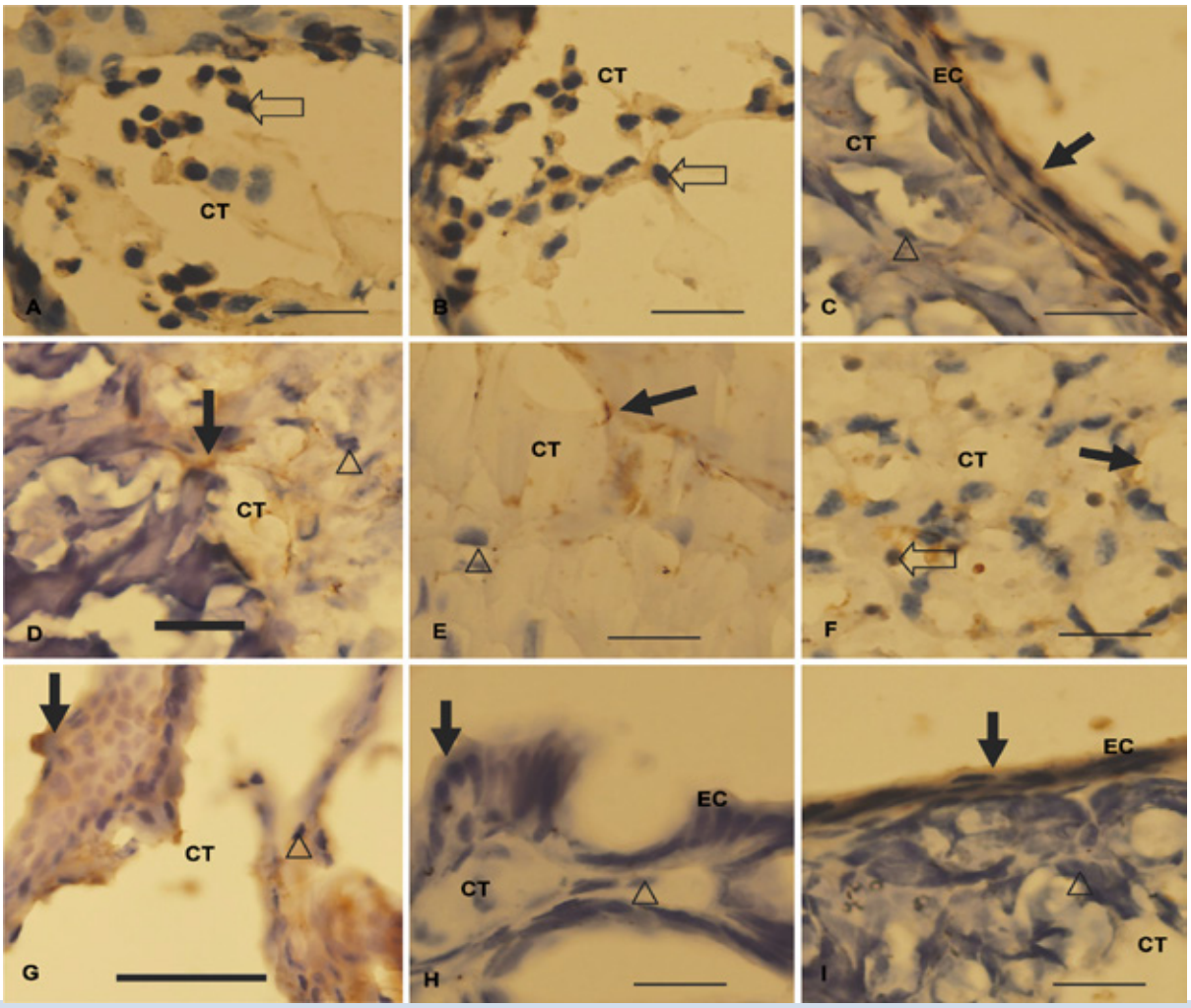

Fig.2 Distribution of delta opioid receptor Immunoreactivity in the respiratory, circulatory, excretory and reproductive systems
A~B. Distribution of delta opioid receptor Immunoreactivity in the branchia; C~D. Distribution of delta opioid receptor Immunoreactivity in the branchial gland; E. Distribution of delta opioid receptor Immunoreactivity in the ventricle; F. Distribution of delta opioid receptor Immunoreactivity in the branchial heart; G. Distribution of delta opioid receptor Immunoreactivity in the kidneys; H. Distribution of delta opioid receptor Immunoreactivity in the ovary; I. Distribution of delta opioid receptor Immunoreactivity in the oviducal gland. Scale bars in G=50μm, the others=20μm.
Black arrows-positive delta opioid receptor granules; white arrows-positive cells; open triangles-karyon; EC-epithelium; CT-connective tissue.

Table 1 Expression of Leu-enk in the Branchia, Ventricle, Branchial Heart and Ovary by ELISA ($\bar{\chi}$±s, n=9)

Tissues	Branchia	Ventricle	Branchial Heart	Ovary
Leu-enk concentration (pg/ml)	8.26±0.94	1.20±0.18	3.15±0.41	0.69±0.07

Expression of Leu-enk in the Branchia, Ventricle, Branchial Heart and Ovary by ELISA

As shown in Table 1, the highest concentration of Leu-enk was located at branchia (8.26±0.94 pg/ml), followed by branchial heart (3.15±0.41 pg/ml). The above mentionned organs are both related to the respiration of *O. ocellatus*. On the contrary, Leu-enk concentration was very low in both ventricles (1.20±0.18 pg/ml) and ovaries (0.69±0.07 pg/ml).

DISCUSSION

Enk and opioid receptors have been observed in the circulatory system of mollusk. Stefano and Catapane (1979) have detected the leu-enkephalin-like and the met-enkephalin-like in the pericardial cavity of *M. edulis*. Using a highly specific antibody, Martin et al. (1979) detected immunoreactivity of met-enkephalin-like in large granules of numerous distinct cells that are embedded in a layer of secretory terminals

inside the vena cava of *Octopus variabilis*. Using indirect immunocytochemistry, Ewadinger et al. (1996) found immunoreactive individual cells and cell clusters of met-enkephalin-like in the CNS, as well as to fibers in the atrium of the heart of the freshwater snail *Lymnaea stagnalis*. Using immunohistochemistry, the immunoreactivities of leu-enkephalin-like, δ, μ, and κ opioid receptors were localized in haemolymph of the scallop *Chlamys farreri* (Liu 2008; Liu and Sun 2010). In our study, the ELISA results indicated the presence of Leu-enk in the ventricle (1.20±0.18 pg/ml) and branchial heart (3.15±0.41 pg/ml) of *O. ocellatus*. In addition, the immunohistochemical method localized both δ opioid receptor and Leu-enk in the epithelium and connective tissue of ventricle, as well as in the connective tissue of branchial heart. However, the muscle tissue was found immunonegative for both δ opioid receptor and Leu-enk, suggesting that further studies are required in order to clarify their contribution in the regulation of the *O. ocellatus*' spontaneous rhythmicity and myocardial contractility.

Mantione et al. (2006) found that morphine regulated gill ciliary activity via binding with μ3 opiate receptor and nitric oxide released in *M. edulis*. The results of Liu and Sun (2010) demonstrated that μ, δ, and κ opioid-like receptors were present in the central axis and gill filaments of the scallop *C. farreri*, indicating that endogenous opioid peptides and receptors may play a significant role on controlling of ciliary activity of the scallop. Our results are consistent with the above results. In our study, Leu-enk was detected using ELISA method in the branchia (8.26±0.94 pg/ml) and branchial heart (3.15±0.41 pg/ml) of *O. ocellatus*. Moreover, the immunohistochemical method localized both δ opioid receptor and Leu-enk in the respiratory epithelium and connective tissue of branchia, as well as in the connective tissue of branchial heart. Our results are further supporting the role of Leu-enk via coupling with δ opioid receptor on controlling of ciliary activity, herein observed in the *O. ocellatus*.

In the present study, Leu-enk was detected in the ovary using ELISA and immunohistochemical method. In addition, δ opioid receptor immunopositivity was also observed in the epithelial tissue of the ovary of *O. ocellatus*. Moreover, δ opioid receptor and Leu-enk immunoreactivity were also localized in the gland cells of the oviducal gland. Since there are no available data regarding Leu-enk and opioid receptors in the reproductive system of cephalopods, the present study herein demonstrates their presence, suggesting that Leu-enk may play a weak role in reproduction of *O. ocellatus*, via coupling to δ opioid receptor. Further studies are required about the specific role and mechanism of δ opioid receptor and Leu-enk in the ovary and oviducal gland of *O. ocellatus*, and whether Leu-enk and opioid receptors are presented in the reproductive system of other cephalopods. The densities of δ opioid receptor and Leu-enk may be related to the physiological functions in different parts of *O. ocellatus*, awaiting further study in future. The absence of immunoreactivities of κ and μ opioid receptors in the respiratory, circulatory, excretory and reproductive systems of *O. ocellatus*, indicates that Enk has weak μ and κ opioid receptors selectivity and further supports its consideration as the endogenous ligand of δ opioid receptor.

Endogenous opioid peptides have been shown to be involved in the mobilization, directed movement, adherence of immunoreactive cells and in several related immune processes via coupling to opioid receptors. There is evidence for the presence of opioids in lymphoid cells of *M. edulis* and human and for their release into the circulation in response to stress (Hughes et al. 1991; Stefano et al. 1990, 1993b, 1995). In invertebrates, these same peptides induce chemotaxis and the release of mammalianlike cytokines, including tumor necrosis factor-α and interleukin-1, -6 (Stefano et al. 1991a, 1991b; Osman et al. 2003). By combining with δ opioid receptor, Enk could up-regulated the immunity of the mollusk (Stefano et al. 1993a). In our previous study, we found that the immunoreactivities of δ opioid receptor and Leu-enk were presented in the mantles and feet of *O. ocellatus* (Sha et al. 2012). The mantles and feet, besides their relation to animal movement, are also related to mucosal immunity and external defense of *O. ocellatus*, since they are in direct contact with the external environment (Wang et al. 2005). In the present study, the immunoreactivities of δ opioid receptor and Leu-enk were additionally presented in the branchia and branchial heart of *O. ocellatus*. These results may indicate that the δ opioid receptor and Leu-enk in these regions could also participate in the mucosal immunity, mucus secretion, and external defense of *O. ocellatus*, since these organs are also in direct contact with seawater.

In conclusion, the results showed that δ opioid receptor and Leu-enk immunoreactivities were both detected in the branchia, ventricle, branchial heart,

ovary and oviducal gland, while δ opioid receptor was additionally detected in the branchial gland and kidneys. The majority of the examined tissues presented weak immunoreactivities of δ opioid receptor and Leu-enk and the quantitative comparison results of Leu-enk ELISA in branchia, ventricle, branchial heart and ovary were consistent with the results of immunohistochemistry. The combined results of δ opioid receptor and Leu-enk immunoreactivities indicated that they may be involved in the regulations of respiration, circulation, reproduction and endocrine likely along with other hormones or neurotransmitters in the *O. ocellatus*' body.

CONFLICT OF INTEREST

The authors have declared that there were no conflicts of interest.

ACKNOWLEDGMENTS

The authors thank Yiyan Wang and Hushan Sun for their valuable discussion and for reading the article.

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