Global Journal of Biotechnology & Biochemistry 7 (4): 110-114, 2012
ISSN 2078-466X

DOI: 10.5829/idosi.gjbb.2012.7.4.65126

Stimulation of Ovarian Maturation in Freshwater Crab, *Oziotelphusa senex senex* Fabricius by Leucine Enkephalin

[1]B. Kishori, [2]P. Ramachandra Reddy and [3]P. Sreenivasula Reddy

[1]Department of Biotechnology, Sri Padmavati Mahila Visvavidyalayam
(Women's University), Tirupati - 517 502, A.P., India
[2]Department of Biochemistry, Yogi Vemana University, Kadapa- 516 003, A.P., India
[3]Department of Zoology, Sri Venkateswara University, Tirupati - 517 502, A.P., India

Abstract: Enkephalins are the peptide neurotransmitters, also play a role in the crustacean reproduction. In the present study administration of leucine enkephalin at the dose of 10^{-8} mole/crab on 1st, 7th, 14th and 21st day of experiment resulted in significant increase in ovarian growth of freshwater crab *Oziotelphusa senex senex* Febricius. These results provide evidence to support the hypothesis that enkephalins act as gonadotropins in crustaceans.

Key words: Ovarian Index · Leucine Enkephalin · Histological Studies · Crab

INTRODUCTION

Since the discovery of opioid peptides in crustaceans [1], many studies were conducted to demonstrate the occurrence of these peptides in several crustaceans [2]. Even though there is a large body of immunological evidence for the presence of opioid peptides in crustaceans, relatively few data have been provided concerning their likely physiological activities. Among the described functions of enkephalins are excitatory action for locomotor activity [3], an increased escape response of *Gecarcinus lateralis*, dark adaptation of distal retinal pigment of *Uca pugilator* [4], regulation of hemolymph sugar level in crabs, *Oziotelphusa senex senex* [5], *Scylla serrata* [6] and in prawns *Penaeus indicus* and *Metapenaeus monoceros* [7] and also they act as neurotransmitters.

Methionine enkephalin stimulated release of a red-pigment concentrating hormone and a black-pigment concentrating hormone isolated from eyestalk tissues of *Uca pugilator* [4]. In the same crab, Kulkarni and Fingerman [8] found that the enkephalin stimulated release of the distal retinal pigment dark-adapting hormone [8]. In *Carcinus maenas*, leucine enkephalin inhibited release of crustacean hyperglycemic hormone [9]. However there are no reports on the effects of leucine enkephalin on the mobilization of other neuro-hormones of the sinus gland.

In view of this an elaborate programme was initiated in our laboratory to evaluate the physiological role(s) of enkephalins in crustaceans. We have found a neurotransmitter role for methionine enkephalin in regulating hemolymph sugar level in fresh water crab, *Oziotelphusa senex senex* [5]. Methionine enkephalin induces hyperglycemia through eyestalk hormone in the estuarine crab, *Scylla serrata* [6] and in prawns *Penaeus indicus* and *Metapenaeus monoceros* [7]. The present report is a part of the above mentioned programme and determines the role of leucine enkephalin in regulating the ovarian maturation in fresh water crab *Oziotelphusa senex senex.*

MATERIALS AND METHODS

Intact, inter molt (Stage C4) adult female *Oziotelphusa senex* (body weight 30±2g) were collected from the rice fields and irrigation canals around Tirupati, India. They were acclimatized to the laboratory conditions (temperature 27±1°C; Relative Humidity 75% and a light period 12 h) for 7 days in large glass aquaria partially filled

Corresponding Author: B. Kishori, Department of Biotechnology, Sri Padmavati Mahila Visvavidyalayam (Women's University), Tirupati - 517 502, A.P., India.

with tap water. The crabs were fed *ad libitum* once in every two days with sheep meat and transferred to fresh medium every day.

Leucine enkephalin was purchased from Sigma Chemical Company and dissolved in crustacean saline [10]. In the present study, 75 crabs having a white (immature) ovary were selected and divided into three groups each consisting of 25 crabs. The crabs in the first group served as initial control did not receive any treatment and was sacrificed on the first day of experiment. The crabs in the second group served as concurrent control and was treated the same as the experimental group but received injections of crustacean saline only [10]. The crabs in the third group received injections of 10^{-8} mol/crab of leucine enkephalin in 10 µl volume per injection. The injections were given on the 1st, 7th, 14th and 21st day through the arthrodial membrane at the base of the coxa of the third pair of walking legs and the crabs in the groups 2 and 3 were sacrificed on day 30. After sacrifice, the crabs were weighed and the ovaries were dissected out from the animals and the ovarian indexes were determined using the following formula:

$$\text{Ovarian Index} = \frac{\text{Wet weight of the ovary}}{\text{Wet weight of the crab}} \text{x} 100$$

The ovaries were fixed in aqueous Bouin's fluid. After 24 h fixation, the ovaries were dehydrated through an alcohol series, cleared in xylene and then embedded in paraffin (melting point 56-58°C). Serial sections (7 µm) were cut and stained with haematoxylin and counter stained with eosin. The sections were photographed using phase contrast microscope. The data were analyzed using ANOVA followed by Dunnett's test to determine the level of significance.

RESULTS

In *Oziotelphusa senex senex* the immature ovary is translucent to opaque white in color. During early vitellogenesis (Stage I) the gonad color changes from white to yellow. At this stage the yolk globules are deposited in oocytes at the periphery and the oocyte contains prominent nucleus and nucleolus. During late vitellogenesis (Stage II), peripherally located yolk globules moves towards the centre, gradually replacing protein yolk. At this stage, the color of the gonad becomes light orange red. During oogenesis, the oocyte contains a large accumulation of yolk globules occupying the whole of the oocyte and the color of the ovary becomes dark brown or bright orange.

The ovaries were white and the average ovarian index was 0.512 in initial control crabs. The mean ovarian index of concurrent control crabs was 0.514 which is 0.36% more from the initial control group (Table 1) and morphologically ovaries are white in color (Photograph 1A), whereas the mean ovarian index of the leucine

Table 1: Effect of injection of leucine enkephalin on color of the ovary and ovarian index of the freshwater rice field crab, *Oziotelphusa senex senex* Fabricius

Group	Color of the ovary	Ovarian index
Initial control	White	0.512^{a}±0.008
Concurrent control	White	0.514^{a}±0.010 (0.39%)
Leucine enkephalin injected	Dark brown/bright orange	1.137±0.025 (122.07%)

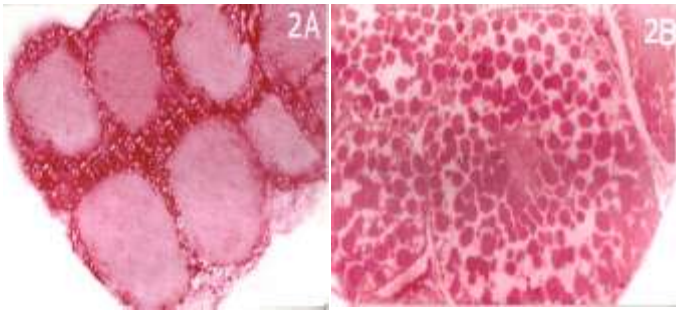
P <0.001

Values are man ± SD (n=25)

Values in parenthesis are percent change from initial control

Values with the same superscript (^{a}) are not significant

Photograph 1: A) Female reproductive system of concurrent control crab showing immature (White) ovary
B) Female reproductive system of leucine enkephalin injected crab showing fully matured (Brown color) ovary



Photograph 2: A) Transverse section of ovary from concurrent control crab showing previtellogenic oocytes surrounded with thick ovarian wall. Scale line = 4 µm.
B) Transverse section of ovary from leucine enkephalin injected crab showing lipovitellin yolk globules occupying the whole oocytes. Scale line = 10µm.

enkephalin injected crabs were significantly ($p<0.001$) higher (1.137) with an increased percent of 122.03 than the corresponding values of the initial control crabs. The crabs in group III had dark brown to bright orange color ovary (Table 1; Photograph 1B). Histological section of the ovary from the concurrent control crabs had a thick ovarian wall with a centrally located germanium surrounded by a number of oocytes (Fig. 2A). Whereas oocytes from crabs treated with leucine enkephalin contain a large accumulation of yolk globules occupying the whole of the oocyte (Fig. 2B).

DISCUSSION

In crustaceans, maturation of the ovary can be identified based upon morphological characteristics and coloration. Increase in size as the oocyte proliferation, increase in diameter due to yolk accumulation and change in color from opaque white to dark brown or orange red due to deposition of carotinoids were used as criteria to determine ovarian maturation by several scientists [11]. The concentration of vitellogenin in the hemolymph an accumulation of yolk globules in oocytes was also used as criteria to determine the ovarian maturation [12]. Recently measurement of ovarian vitellogenin levels at different stages of vary was also used to determine the reproduction with anti vitellin anti-sera [13]. The present study focused on the effect of leucine enkephalin on ovarian maturation in female fresh water rice field crab *Oziotelphusa senex senex* measuring the weight of the ovary in relation to the body weight (ovarian index), color of the ovary and also histological change in the deposition of yolk in oocytes.

The comparison of the present data raised for the initial control crabs and the concurrent control crabs indicating that the ovaries are developing slowly but not significantly during the course of experimentation. Injection of leucine enkephalin in to female crabs resulted in significant increase in ovarian index and significant histological change in the deposition of yolk in the ovaries in comparison with concurrent control ovarian histology, indicating the involvement of leucine enkephalin in regulating reproduction in crustaceans.

Evidence for a role played by hormones in regulating crustacean reproduction is accumulating. In decapoda crustaceans, ovarian development is under the control of two neuropeptide hormones known as the gonad-inhibiting hormone (GIH) which is synthesized and secreted from medulla terminalis X-organ sinus gland complex present in the eyestalks [14, 15] and the gonad stimulating hormone (GSH) secreted from the brain and thoracic ganglia [16-20]. Besides these, methyl farnesoate (MF) secreted by mandibular organs is also involved in the stimulation of ovarian development [21-29]. Recent studies also indicate that different biogenic amines are also involved in the regulation of crustacean reproduction [30-33].

Our previous studies established the presence of gonad inhibiting hormone, gonad stimulating hormone and methyl farnesoate in *Oziotelphusa senex senex* [24, 27]. Using immunodiagnostic studies it was demonstrated that the presence of leucine enkephalin in the neuroendocrine complex of the eyestalks of several crustacean [2]. Enkephalins mediated release of neurohormones in crustaceans was also established. It was found that leucine enkephalin induces hypoglycaemia in intact *Uca pugilator* but not in eyestalk less individuals. The contrast activity of leucine enkephalin was hypothesized and proved in the red swamp crayfish *Procambarus clarkii* [34]. In view of the

hypothesis proposed experiments was performed and found the inhibitory action of leucine enkephalin on the release of neurohormones(s) from eyestalks.

With the results of the present study we suggest that the induced ovarian growth in the female crab by the leucine enkephalin might be due to inhibition of release of the gonad inhibiting hormone from eyestalks. Further experiments are in progress in this laboratory to firmly establish the role of leucine enkephalin in inducing reproduction in crab *Oziotelphusa senex senex,* since relatively large amounts of opioid peptides are present in the brain and thoracic ganglia [35, 36], where gonad stimulating hormone is synthesized.

ACKNOWLEDGEMENTS

We are grateful to Department of Science and Technology, New Delhi for financial support in the form of research grants (SP/SO/CO4/96 and PS/SO/C42/20010) to PSR. We thank Dr. T. Pushpalatha for valuable technical assistance in taking photographs and Mr. S. Umasankar for maintaining the crabs in the laboratory.

REFERENCES

1. Mancillas, J.R., J.F. Mc Ginty, A.I. Selverston, H. Karten and F.E. Blook, 1981. Immunocytological localization of enkephalin and substance P in retina and eyestalk neurons of lobster. Nat., 293: 576-578.
2. Nagabhushanam, R., R. Sarojini, P.S. Reddy, S. Devi and M. Fingerman, 1995. Opioid peptides in invertebrates: Localization, distribution and possible functional roles. Curr. Sci., 69: 659-671.
3. Martinez, E.A., D. Vassell and G.B. Stefano, 1986. Opioid potentiated chromatophorotropin regulation of pigment migration in the land crab, *Gecarnius lateralis*. Comp. Biochem. Physiol., 83: 77-82.
4. Quackenbush, L.S. and M. Fingerman, 1984. Regulation of neurohormone release in the fiddler crab, *Uca pugilator*: Effect of gamma-aminobutyric acid, octopamine, Met-enkephalin and beta-endorphin. Comp. Biochem. Physiol., 79: 77-84.
5. Reddy, P.S., 1999. A neurotransmitter role for methionine-enkephalin in causing hyperglycemia in the fresh water crab, *Oziotelphusa senex senex*, Curr. Sci., 76: 1126-1128.
6. Reddy, P.S. and B. Kishori, 2001. Methionine-enkephalin induces hyperglycemia through eyestalk hormone in the estuarine crab, *Scylla serrata.* Biol. Bull., 201: 17-25.
7. Kishori, B., B. Prema Sheela, R. Ramamurthi and P.S. Reddy, 2001. Evidence for hyperglycemic effect of methionine–enkephalin in the prawns *Penaeus indicus* and *Metapenaeus monocerus.* Gen. Comp. Endocrinol., 123: 90-99.
8. Kulkarni, G.K. and M. Fingerman, 1987. Distal retinal pigments of the fiddler crab, *Uca pugilator*: Release of the dark adapting hormone by methionine enkephalin and FMRF amide. Pig. Cell Res., 1: 51-56.
9. Rothe, H., W. Luschen, A. Asken, A. Willling and P.P. Jaros, 1991. Purified crustacean enkephalin inhibits release of hyperglycemic hormone in the crab *Carcinus maenas* L. Comp. Biochem. Physiol., 99: 57-62.
10. Van Harreveld, A., 1963. A Physiological solution for fresh water crustaceans. Proceedings of the society for Experimental Biology and Medicine. New York, 34: 428-432.
11. Charniaxux-Cotton, H. and G.G. Payen, 1988 Crustacean reproduction In: *Endocrinology invertebrate types.* (ed. By H. Laufer and R.G.H. Downer), pp: 279-303. Aln. Liss. Inc. New York.
12. Tsukimura, B., 2001. Crustacean vitellogenesis: Its role in oocyte development. American Zoologist, 41: 465-476.
13. Reddy, P.R., P. Kiranmayi, K. Thanuja Kumari and P.S. Reddy, 2006. 17α-hydroxyprogesterone induced ovarian growth and vitellogenesis in the freshwater rice field crab *Oziotelphusa senex senex*. Aquaculture, 254: 768-775.
14. Adiyodi, K.G. and R.G. Adiyodi, 1970. Endocrine control of reproduction in decapod crustacean. Biol. Rev., 45: 121-165.
15. Nagabhushanam, R., G.K. Kulkarni and R. Sarojini, 1980. Reproductive endocrinology of dacapod crustacean. National Academy Science. Golden Jubilee Commemoration, pp: 1-38.
16. Otsu, T., 1963. Biohormonal control of sexual cycle in the fresh water crab, *Potamon dehaani*. Embryol., 8: 1-20.
17. Gomez, R., 1965. Acceleration of development of gonads by implantation of brain in the crab *Paratelphusa hydrodromous.* Naturwissenschaften, 52: 216.
18. Eastman-Reks, S. and M. Fingerman, 1984. Effects of neuroendocrine tissue and cyclic AMP on ovarian growth *in vivo* and *in vitro* in the fiddler crab, *Uca pugilator.* Comp. Biochem. Physio., 79: 679-684.

19. Kulkarni, G.K., R. Nagabhushanam, G. Amaldoss, R.G. Jaiswal and M. Fingerman, 1992. *In vivo* stimulation of ovarian development in the red swamp crayfish *Procambarus clarkia* (Girard) by 5-hydroxytryptamine. Invert. Reprod. Develop., 21: 231-240.
20. Yano, I., 1992. Effect of thoracic ganglion on vitellogenin secretion in kuruma prawn, *Penaeus japonicus.* Bulletin National Research Institute of Aquaculture, 21: 9-14.
21. Hinsch, G.W., 1980. Effects of mandibular gland implants upon spider crab ovary. Tran. Amer. Micro. Soc., 99: 317-322.
22. Laufer, H.S, 1992. Method for increasing crustacean larval production. United States Patent., 5: 481.
23. Laufer, H., W.J. Biggers and J.S.B. Ahl, 1998. Stimulation of ovarian maturation in the crayfish *Procambarus clarkii* by methyl farnesoate. Gen. Comp. Endocrinol., 111: 113-118.
24. Reddy, P.S. and R. Ramamurthi, 1998. Methyl farnesoate stimulates ovarian maturation in the fresh water crab, *Oiotelphusa senex.* Curr. Sci., 74: 68-70.
25. Reddy, P.S. and R. Ramamurthi, 1999. Recent trends in crustacean endocrine research. Proc. Ind. Nat. Sci. Acad., 65: 15-32.
26. Rotllant, G., P. Takac, L. Liu, G.L. Scott and H. Laufer, 2000. Role of ecdysteriods and methyl farnesoate in morphogenesis and terminal molt in polymorphic males of spider crab, *Libinia emarginata.* Aquaculture, 190: 103-118.
27. Rodrigquez, E.M., L.S. Lopez Greco, D.A. Medesani, H. Laufer and M. Fingerman, 2002. Effect of methyl farnesoate, alone and in combination with other hormones, on ovarian growth of the red swamp crayfish, *Procambarus clarkii,* during vitellogenesis. Gen. Comp. Endocrinol., 125: 34-40.
28. Nagaraju, G.P.C., N.J. Suraj and P.S. Reddy, 2004. Methyl farnesoate stimulates gonad development in *Macrobrachium malcolmsonii* (H. Milne Edwards) (Decapoda, Palemonidae), *Crustaceana,* 76: 1171-1178.
29. Suneetha, Y., P. Naga Jyothi and M. Srinivasulu Reddy, 2010. Impact of methyl farnesoate in the regulation of molting and reproduction in the tropical penaeid prawn *Penaueu monodon*. Glob. J. Biotechnol. Biochem., 5(4): 226-230.
30. Richardson, H.G., M. Deecaraman and M. Fingerman, 1991. The effect of biogenic amines on ovarian development in the fiddler crab, *Uca pugilator.* Comp. Biochem. Physiol., 99: 53-56.
31. Kulkarni, G.K., L. Glade and M. Fingerman, 1991. Oogenesis and effects of neuroendocrine tissues on synthesis of protein by the ovary of the red swamp crayfish *Procambarus clarkii* (Girard). J. Crust. Biol., 11: 513-522.
32. Sarojini, R., R. Nagabhushanam and M. Fingerman, 1995a. Mode of action of the neurotransmitter 5-hydroxytryptamine in stimulating ovarian maturation in the red swamp crayfish, *Procambarus clarkii.* An *in invo* and *in vitro* study. J. Exp. Zool., 271: 395-400.
33. Vaca, A.A. and Jorge Alfaro, 2000. Ovarian maturation and spawning in the white shrimp, *penaeus vannamei,* by serotonin injection, Aquaculture, 182: 373-385.
34. Sarojini, R., R. Nagabhushanam and M. Fingerman, 1995b. Dopaminergic and enkephalinergic involvement in the regulation of blood glucose in the red swamp crayfish *Procambarus clarkii.* Gen. Comp. Endocrinol., 97: 160-170.
35. Dircksen, H., 1990. Immunocytochemical identification of the neurosecretory products of pericardial organs of *Carcinus maenas.* In: Frontiers in Crustacean Neurobiology (ed. By K. Wiese, W.D. Krenz, J.H. Tautz, H. Reichert and B. Mullony), pp: 485-491. Birkhauser, Basel.
36. Luschen, W., F. Buck, A. Willing and P.P. Jaros, 1991. Isolation, sequence analysis and physiological properties of enkephalins in the nervous system of the shore crab, *Carcinus maenas.* L. Proc. Ind. Nat. Sci. Acad., USA, 88: 8671-8675.