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RESEARCH ARTICLE

CRUSTACEAN CARDIOACTIVE PEPTIDE (CCAP) AND CORAZONIN (CRZ) AS PUTATIVE CIRCADIAN CLOCK OUTPUT SIGNALS IN THE CENTRAL NERVOUS SYSTEM OF THE TERRESTRIAL ISOPOD, *ARMADILLIDIUM VULGARE* (LATREILLE).

Maged M. A. Fouda[1], Moustafa M. H. Sarhan[1] and Makio Takeda[2].

1. Zoology Dept., Faculty of Science, Al-Azhar University, Assuit branch, Assuit, Egypt. |
2. Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657 8501, Japan.

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Corresponding Author

Maged M. A. Fouda.

Abstract

Crustacean cardioactive peptide (CCAP)- and corazonin (Crz)-like immunohistochemical reactivity (CCAP-ir and Crz-ir) occurred in small sets of neurons in the cephalic ganglia of the isopod, *Armadillidium vulgare*. The distribution patterns of both reacitivities were similar in the optic lobe (OL), protocerebrum (PC), tritocerebrum (TC) and suboesophageal mass (SM). Also, these reactivates were co-localized with circadian clock related gene products (CYC and CLK) in the OL and SM; 5 CCAP-ir cells occurred in each OL, weak signals in the pseudofrontal organ (PFO), 2 cells in the PC, one cell in the accessory lobe (AL), a pair of cells in the TC and 10 cells in the SM, while Crz-ir occurred in OL, 12 cells in each lobe. PFO was strongly stained, one cell in PC, pair of cells in TC and 2 pairs of cells in SM one on each side. Double-labeling experiments also showed that some of the detected cells reacted with clock proteins, cycle (CYC) and clock (CLK). They were colocalized with both CCAP-ir and Crz-ir in OL, PFO and the cells of mandibular ganglion (CMD). No difference was found in number or distribution of CCAP-ir or Crz-ir between males and females. The distribution of cells, the projection of immunoreactive fibers to several brain regions, the stomatogastric nervous system and the neurohaemal organs indicated multiple functions of these neuropeptides.

Introduction:-

The neurotransmitters or neurohormonal system in the output pathway of the circadian system have been investigated in a small number of species such as *Drosophila melanogaster* and *Leucophaea maderae* where pigment dispersing factor (PDF) plays an important role in the mutual coupling among the oscillators where PDF-ir and PER-ir were colocalized in the same neurons. However, lepidopterans species do not show this colocalization (Helfrich-Foerster, 1995; Sauman and Reppert, 1996). Recently, we cloned precursor gene of pigment-dispersing hormone (PDH) from *A. vulgare*, (Fouda et al., 2010). PDF, homolog of PDH is putative output neuropeptide of circadian clock neurons in insects (Rao, 2001). We confirmed co-localization of PDH-ir with CLK- and CYC-ir. Based on these results, we hypothesized involvement of PDH in circadian rhythms as neurotransmitter (Fouda et al., 2010).

The crustacean cardioactive peptide (CCAP) was first identified and sequenced in the green shore crab Carcinus maenas as an unusual cyclic nonapeptide, PFCNAFTGC-NH_2 (Stangier et al., 1987). Later, it was found without any amino acid sequence variation in a number of crustaceans and insects (Dircksen, 1998). CCAP involved in modulation of stomatogastric ganglion motor patterns in crustaceans (Weimann et al., 1997; Richards and

Marder, 2000). In cray fish and crabs CCAP increased during moulting (Phlippen et al., 2000) and it may play a role in reproduction (Chung et al., 2006). In insects CCAP has several identified functions as cardioacceleration (Tublitz and Truman, 1985a,b; Tublitz and Evans, 1986; Cheung et al., 1992); modulation of hindgut activity (Stangier and Keller, 1990; Stangier, 1991; Tublitz et al.,1992), acceleration of blood circulation during wing inflation (Tublitz and Truman, 1985 b), modulation of oviduct contraction in *Manduca sexta* (Marhall and Reynolds, 1998) and *Locusta migratoria* (Donini et al., 2001; Donini and Lange, 2002), a secretagogue action in *L. migratoria* (Veelaert et al., 1997) and plays a significant role in the proximal triggering of ecdysis (Gammie and Truman, 1997). A region of the accessory medulla of the locust *L. migratoria* (Dircksen and Homberg, 1995) and in cephalic ganglia of *Dianemobius nigrofasciatus* and *Allonemobius allardi* (Sehadová et al., 2007) showed CCAP-ir. CCAP-ir was expressed in a series of neurons in the ventral nerve cord of several Holometabola involved in the regulation of ecdysial behaviour (Truman, 2005). CCAP-ir is distributed throughout the CNS of crayfish (Trube et al., 1991 a,b), and that of crab (Dircksen and Keller, 1988). CCAP-ir neurons in the ventral nerve cord of *D. melanogaster* contain the RNA-binding protein LARK, which has been proposed to function as a regulatory element in the circadian clock output pathway (McNeil et al., 1998). In cricket *D. nigrofasciatus*, CCAP-ir was established in two of the six large PER-ir cells in the Pfv cluster (Sehadová et al., 2007).

Corazonin (Crz), an undecapeptide, has been identified in the nervous system and hemolymph of various insect and crustacean species. Several Crz isoforms have been characterized: [Arg^7]-Crz and [His^7]-Crz (Veenstra, 1989, 1991). Both isoforms have been identified in a great number of insect species (Veenstra, 1991; Hua et al., 2000). Also there are other isoforms as [Thr^4, His^7]- Crz (Verleyen et al., 2006), [Tyr^3, Gln^7, Gln^{10}]- Crz. [His^4, Gln^7]- Crz, [Gln^{10}]- Crz (Predel et al., 2007). Corazonin elicits a retraction of erythrophore pigment granules and dispersion of leucophore pigment granules in the crayfish *Procambarus clarkii*. The effects are dose-dependent. [His^7]-Crz-ir somata and fibers were identified in various regions of the eyestalk (medulla terminalis, medulla interna and medulla externa) with the anti-[His^7]-Crz antibody. These results suggest the possible existence of a corazonin-like peptide in crustaceans (Porras et al., 2003). [Arg^7]-Crz was discovered from the American grasshopper, *Schistocerca americana*, as a cardioaccelator (Veenstra, 1991). It has received much attention as an important hormone controlling phase-related darkening and morphogenesis (Hasegawa and Tanaka, 1994; Tawfik et al., 1999; Tanaka, 2000 a,b, 2001, 2004; Breuer et al., 2003). Crzs stimulate the heartbeat, and [Arg^7]-Crz also induces contractions in isolated visceral muscles, such as the antennal heart, hiperneural muscle, less in the muscles of the foregut and none in those of the oviduct and hindgut of *Periplaneta americana* (Predel, 2001; Predel et al., 1994). The Crz is implicated to the circadian clock and used as a signal transmitting the circadian timing command for eclosion (Kim et al., 2004). The relationship of Crz to the circadian clock has been deduced from immunocytochemical studies. For example, in cockroach (Petri et al., 1995), in locust and a grasshopper (Roller et al., 2003) and in *Bombyx mori* (Shao et al., 2003), Crz-immunoreactive (Crz-ir) fibers ramify in the accessory medulla of optic lobes (OL) in which the clock protein Period (PER) is expressed (Lupien et al., 2003). [Arg^7]-Crz-ir in the brain of *Manduca sexta* is restricted to four pairs of Ia1 cells that co-express PER (Wise et al., 2002). [Arg^7]-Crz-ir (Hansen et al., 2001; Roller et al., 2003) and several clock proteins (Sauman and Reppert, 1996; Sehadová et al., 2004) have been found in homologous cells in other moth species but no co-localization has been demonstrated. Crz-ir occurs in small sets of neurons in the cephalic ganglia of *D. nigrofasciatus* and *A. allardi* (Sehadová et al., 2007), Interaction between the circadian clock and Crz has also been proposed to occur in *D. melanogaster* (Choi et al., 2005). In *D. nigrofasciatus* double-staining showed that the Crz-ir perikarya and axons in the dorsolateral brain were located in close vicinity to the PER-ir cells and their neurites (Sehadová et al., 2007). We have shown that CLK, CYC and PER were localized in brain of *A. vulgare* (Fouda, 2010). In the current study the distribution of Crz-ir and CCAP-ir was examined.

Materials and Methods:-

Animals and sample preparation:-

Adults of *A. vulgare* were collected on Rokkodai campus of Kobe university, Japan (34^o 73 N and 135^o 23 E), and kept at 25^oC under LD 12:12 h for at least 7 days before they were dissected in the middle of the photophase (between ZT 4 and ZT 8, where ZT, Zeitgeber Time, is designated by hours after lights-on of LD 12:12).

Immunocytochemistry:-

Whole heads were separated from anesthetized animals in sterile saline (PBS), fixed immediately and kept overnight at 4°C in Bouin's solution (15 vol picric acid, 5 vol formalin, 1 vol acetic acid). Standard histochemical techniques were employed for tissue dehydration, embedding in paraplast, sectioning (8 μm), deparaffinization in

xylene and rehydration in ethanol. The sections were then washed in distilled water and TRIS-buffered saline (TBS; 135 mM NaCl, 2.6 mM KCl, 25 mM TRIS-HCl, pH 7.6) at room temperature (RT), blocked with 1.5% normal goat serum in TBS-T for 1 h at RT. The sections were incubated with primary antibody, rabbit anti-CCAP or anti-Crz (His^7) antiserum diluted at 1:500, in a humidified chamber overnight at 4°C. In control experiments, the primary antibody was substituted by normal serum. Further processing was conducted at RT. After thorough rinsing with TBS (10min×3), bound antibody was detected with the rabbit IgG VECTASTAIN Elite ABC Kit (Vector Laboratories, Burlingame, CA) diluted at 1:200 in blocking serum for 90 min, washed in TBS (10min×3) and then treated with HRP-labeled avidin-biotin complex (diluted in TBS-0.1%Tw) for 30 min. Following rinsing with TBS (10min×3) and with 0.1M TRIS–HCl, pH 7.5 (5 min), The activity of the HRP conjugated secondary antibody was visualized with 0.005% H_2O_2 and 0.25 mM 3,3'-diaminobenzidine tetrahydrochloride (DAB; in 0.1 M TRIS-HCl, pH 7.5), and the reaction was stopped in distilled water. Stained sections were dehydrated and mounted with Bioleit mounting medium (Kouken Rika, Osaka, Japan) and visualized under a BX50F4 microscope (Olympus, Tokyo, Japan) equipped with Nomarski optics differential interference contrast (DIC) and photographed with a charge-coupled device (CCD) camera (Olympus).

Double-labeling experiments using rat anti-*B.mori* CYC or rat anti-*B.mori* CLK with rabbit anti-CCAP and anti-Crz antisera were conducted as follows. The sections were incubated with a cocktail of primary antibodies against CYC or CLK at 1:125, and CCAP or Crz at 1:250), and then the rat IgG was visualized as a red fluorophore using VECTASTAIN Elite ABC rat IgG Kit (Vector Laboratories) and TSA Labeling Kit #42 with Alexa Fluor® 555 (Invitrogen, Tokyo). The rabbit IgG was visualized as a green fluorophore using goat anti-rabbit IgG conjugated with Alexa Fluor® 488 (Invitrogen). The stained sections were mounted in Aqua-Poly/Mount medium (Polysciences, PA, USA), and examined by using a BX50 microscope (Olympus, Tokyo) equipped with BX-FLA reflected light fluorescence and WIG and NIBA mirror/filter units for the detection of Alexa Fluor 555 and Alexa Fluor 488, respectively.

Antibodies and Specificity of the primary antibody:-

We identified CCAP from *P. Americana* midgut (Sakai et al., 2004) and raised the antibody using KHL as a conjugate (Genemed, California, USA). Specificity was checked with primary antibody blank and preabsorption test (Sakai et al., 2004). Colocalization with clock neurons have been demonstrated in several species (Sehadová et al., 2007). For the detection of Crz, we used a rabbit polyclonal antibody raised against [His^7]-Crz (a gift of Dr. Seiji Tanaka,Wako, Nagano, Japan). This antibody showed almost identical reactivity as [Arg^7]-Crz-ir in *B. mori* brain (Shao et al.,2003). Preabsorption test was performed where each antigen (1 ng/ml) was diluted together with its antibody (anti-CCAP and anti-Crz [His^7] 1:500 in TBS buffer with a ratio of 1:5 and incubated overnight at 4 ^oC). Both normal and preabsorbed serum showed no immunoreactivity. The specificity of anti-*B. mori* CYC (Rat) and anti-*B. mori* CLK (Rat) antibodies were further confirmed by preabsorption test (Shao et al., 2008).

Results:-

Detection of CCAP-ir:-

Fig. 1A illustrates a schematic diagram showing the brain of *A.vulgare* with morphological land marks. CCAP-ir appeared in discrete perikarya and fibers in *A.vulgare* brain (Fig. 1). A wide distribution of CCAP-ir occured in the optic lobe (OL, Fig. 1A-F), accessory lobe (AL, Fig. 1G), protocerebrum (PC; Fig. 1E, F), tritocerebrum (TC, Fig. 1H), and subesophageal mass (SM, Fig. 1 I-L), while no CCAP-ir was observed in the central body (CB), deutocerebrum (DC) and circumesophageal connective (CEC) (Fig. 1A, Table 1). A weak CCAP-ir was observed in the pseudofrontal organ (PFO, Fig 1B, arrows). Two strongly stained small neurons were observed between the medulla interna (MI) and medulla externa (ME) (Fig. 1 C arrow , E arrow head). Strongly stained varicose fibers occurred dorsally in the OL between the medulla terminalis (MT) and MI (Fig 1C,arrow head). Three large immunoreactive neurons were observed dorsally in the OL (Fig. 1D, F; arrows), a large neurons observed in PC (Fig. 1E, arrow). In the central brain, the accessory lobe (AL) harbored a large neuron (Fig. 1G, arrow).The TC harboured a pair of strongly stained neurons (Fig. 1H, arrow). In the SM, CCAP-ir was observed in 8 big neurons in the cells of mandibular ganglion (CMD), a pair of strongly stained neurons on each side laterally located (Fig. 1I, arrowhead) and one ventro-laterally to neuropile of mandible (NpMD) on each side (Fig. 1J, arrowhead), and two centrally located (Fig. 1K, arrowhead). Also in the cells of maxillulary ganglion (CML), 2 CCAP-ir small neurons strongly stained were observed ventral to neuropile of maxillule (NpML; Fig. 1L, arrowhead). No difference was detected in the distribution and intensity of staining between males and females.

Detection of Crz-ir:-

Crz-ir was localized in the brain of *A. vulgare* (Fig. 2). A wide distribution of [His^7]-Crz-ir occurred in the OL, PC, TC and SM, while no Crz-ir was observed in the CB, AL and DC (Fig.2A, Table 1). Strong Crz-ir was observed in PFO (Fig.2B, arrowhead). A bundle of Crz-ir fibers ran through the optic nerve (ON) extending through the ventral side of the OL (Fig. 2B, D; arrow). Three strongly stained small neurons were observed between the MI and ME (Fig.2C, arrowhead) in addition to a large reactive neuron observed dorsally in the OL (Fig. 2C, arrow). Strongly stained fibers runs through OL (Fig. 2D, arrow) and dense varicose fibers dorsally (Fig. 2D, arrowhead). Strongly stained varicose fiber occurred within the MT (Fig. 2 E,arrow) sending strongly immunopositive fiber to the PC (Fig. 2E, arrowhead). Three large immunoreactive neurons were observed dorsally in the OL (Fig. 2F, arrowhead), moderately stained neuron observed in PC (Fig. 2F, arrow). A large strongly reacted neuron dorsally in OL (Fig. 2G, arrowhead), varicose fibers within MT. A large immunoreactive perikaryon strongly stained was observed in posterior side in the OL (Fig. 2 H, arrowhead). A pair of strongly stained small neurons (Fig. 2 I, arrowhead) and one large strongly stained in the dorsal side of OL (Fig. 2 I, arrow) were also observed. There is strongly stained neurite seen extending down through TC to the SM (Fig. 2J, arrowhead). TC harboured a pair of strongly stained neurons (Fig. 2K, arrowhead). In the SM Crz-ir observed in a pair of large neurons that are strongly stained in CMD lateral to NpMD (Fig. 2 L, arrowhead; 4 K, arrow), centrally in NpMD little arborization seen (picture not shown). No difference was detected between males and females.

Table 1:- The number and intensity of immunoreactive cells to anti-CCAP and anti-Corazonin in both hemispheres in the cerebral ganglia of *A. vulgaris*.

Brain subdivision		**CCAP-ir**	**Crz-ir**
Optic Lobe (OL)		Small cells 4 ++++	Small cells 10 ++++
		Big cells 4 +++ Big cells 2 ++	Big cells 6 ++++ Big cells 6 +++ Big cells 2 ++
		Varicose fibers ++++	Varicose fibers ++++
Pseudofrontal organ (PFO)		+	++++
Protocerebrum (PC)		Big cells 2 ++	Big cells 2 ++
Central body (CB)		---	---
Accessory lobe (AL)		Big cells 2 +++	---
Deutocerebrum (DC)		---	---
Tritocerebrum (TC)		Small cells 4 +++	Big cells 4 ++++
subesophageal mass (SM)	**CMD**	Big cells 4 ++++ Big cells 4 ++	4 big ++++
	CML	Small cells 2 ++++	---

Immunoreactivity was quantified as absent (−), weak (+), moderate (++), considerable (+++) and strong (++++)
CMD: cells of mandibular ganglion; CML: cells of maxillulary ganglion.

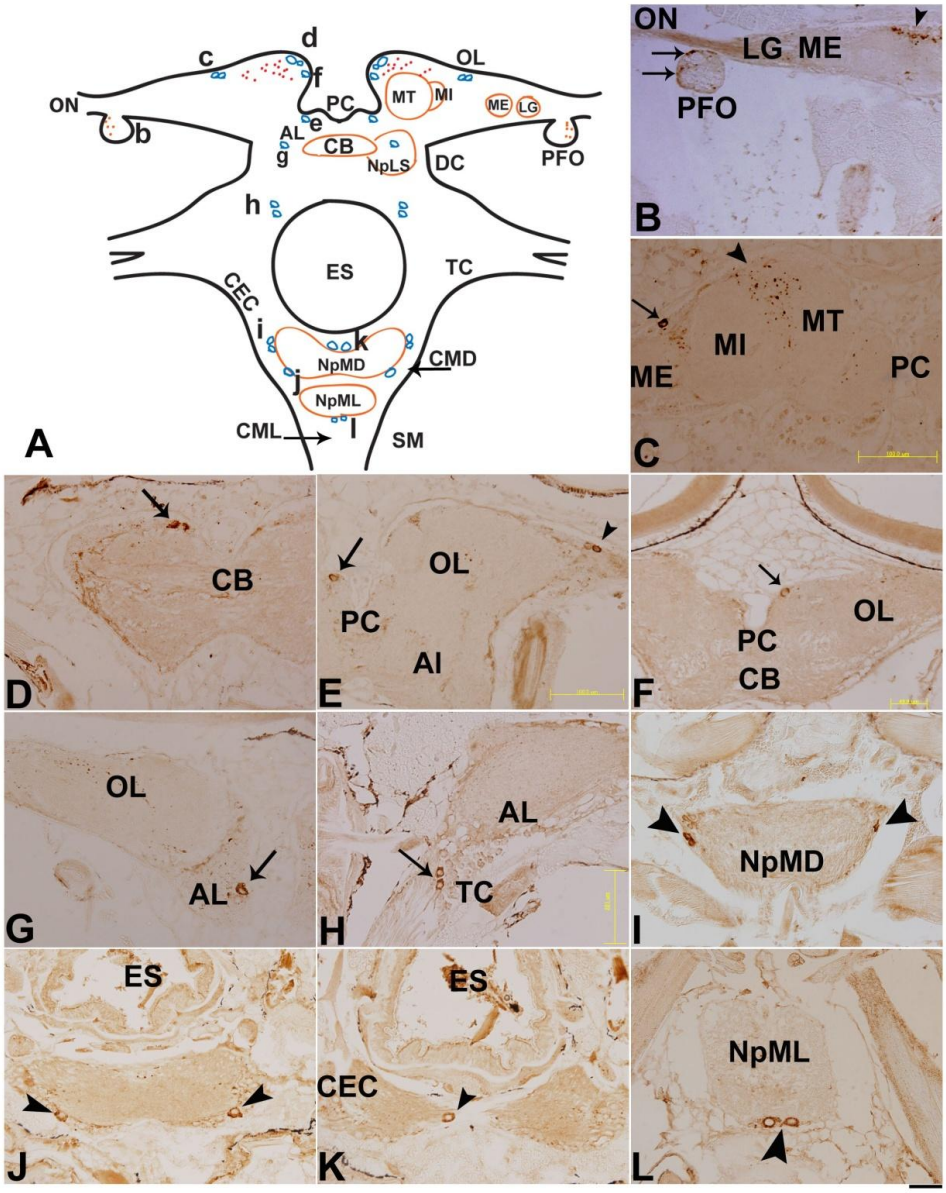

Figure 1:- A schematic diagram illustrating the cephalic neural complex of *A. vulgare* with abbreviations used in the photographs (A) and CCAP-ir in cephalic ganglia of adult *A. vulgare* (b-l) (ON optic nerve, OL optic lobe, PC protocerebrum, MT medulla terminalis, MI medulla interna, ME medulla externa, LG lamina ganglionaris, PFO pseudo-frontal organ, CB central body, AL accessory lobe, DC deutocerebrum, TC tritocerebrum, ES esophagus, CEC circumesophageal connective, SM suboesophageal mass, NpLS neuropile of accessory lobe, NpMD neuropile of mandible, CMD cells of mandibular ganglion, NpML neuropile of maxillule, CML cells of maxillulary ganglion). A. schematic diagram illustrates the number and topography of CCAP-ir neurons and the pathways of their projections (b–l, positions of the respective micrographs in this figure). B. CCAP -ir in PFO (arrows), strongly stained varicose fibers in the OL (arrowhead). C. A strongly stained neuron (dorsally) between the MI and ME, moderately stained varicose fibers dorsally (arrow heads) in the OL. D. A pair of moderately stained neurons dorsally (arrow). E. A large immunoreactive perikaryon in the PC (arrow), small strongly stained neuron (dorsally) between the MI and ME as in C (arrowhead). F. A large immunoreactive perikaryon dorsally in the OL (arrow). G. A positive neuron in the AL (arrow). H. two positive neurons in the TC (arrow). I. A pair of positive neuron lateral to NpMD (arrowhead). J. A positive neuron at the ventro-lateral side of NpMD (arrowhead). K. A strongly stained neuron centrally located (arrowhead). L. Positive neurons ventral to the NpML (arrowhead). Scale bar 50 µm.

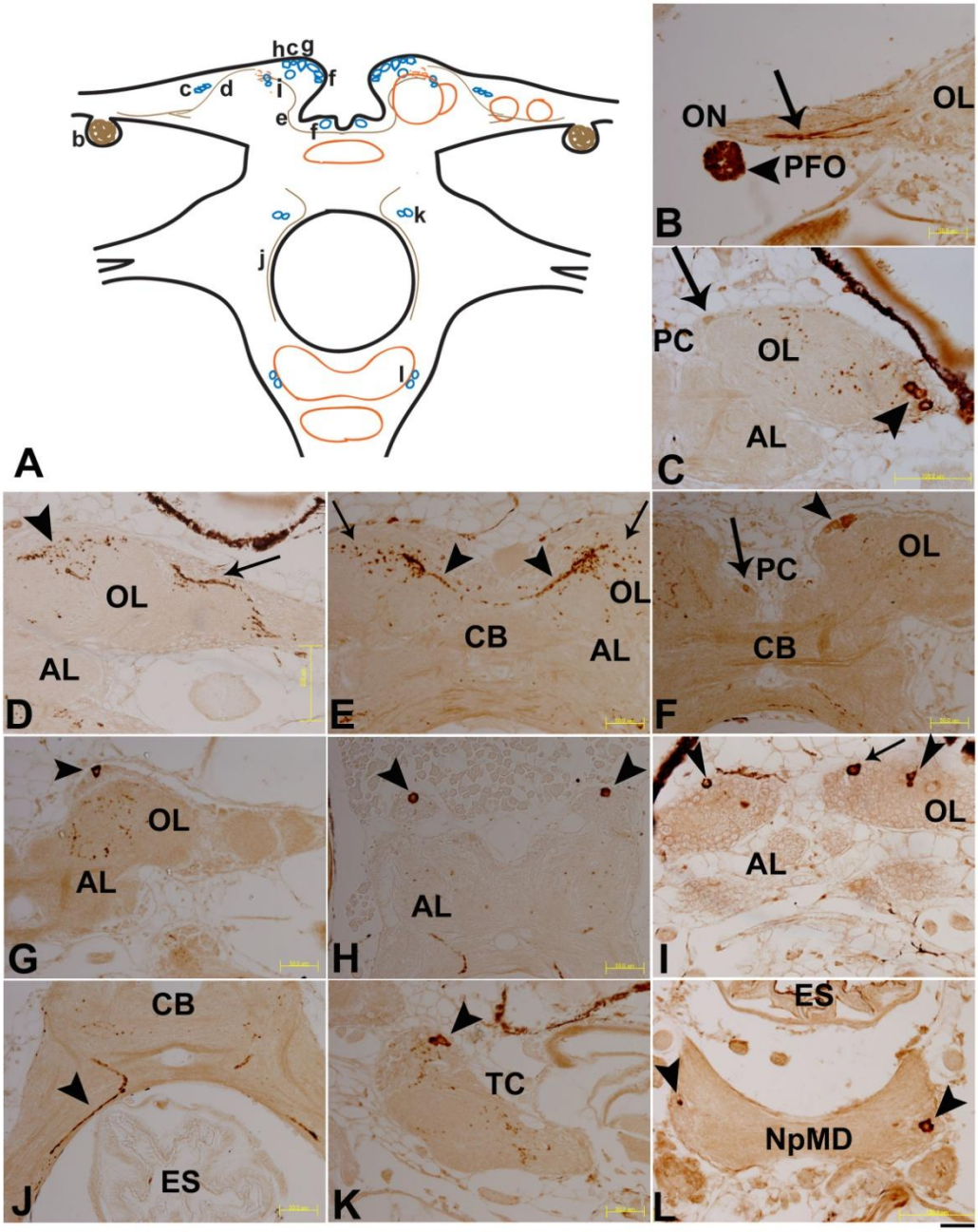

Figure 2:-. Crz-ir in the cephalic ganglia of adult *A.vulgare* A. A schematic diagram illustrating the number and topography of Crz-ir neurons and the pathways of their projections (b–l, positions of the respective micrographs in this figure). B. Crz-ir in PFO (arrowhead), Crz-ir fibers run through the optic nerve (arrow). C. Strongly stained neurons between the MI and ME (arrow head) and weakly stained neuron dorsally in OL (arrow) . D. strongly stained fibers run through OL (arrow) and dense varicose fibers dorsally in OL (arrowhead). E. strongly stained varicose fibers occurred within the MT (arrows) sending strongly immunopositive fiber to the PC (arrowheads). F. Strongly stained neurons dorsally in OL (arrowhead), moderately stained neuron in the PC (arrow). G. A large immunoreactive perikaryon dorsally in OL. H. A large immunoreactive perikaryon (posterior side) in the OL (arrowhead). I. Two strongly stained small neurons (arrow heads), one large strongly stained in the dorsal side of OL (arrow). J. A strongly stained neurite extends ventrally through TC (arrowhead). K. A pair of strongly stained neurons in TC (arrowhead). L. strongly stained neurons lateral to the NpMD (arrowhead). Scale bar 50 µm.

Co-localization of CCAP-ir, CLK-ir, and CYC-ir in the cephalic ganglia of *A. vulgare*:-

Double-labeling with anti-*Pa*-CCAP and *Bm*-CLK antibodies was conducted (Fig. 3 A-C, G-I, M-O), revealing that both CCAP-ir and Clk-ir occurred in 2 small neurons between the MI and ME (Fig. 3 A-C arrows, Fig.1 C, arrow for CCAP), and that PFO showed both weak immunoreactivities, arrow (Fig. 3 M-O, arrows; Fig.1 B, arrow). A bilateral pair of large neurons in the SM adjacent to NpMD were similar in co-localization (Fig. 3G-I arrows, Fig 1 I, arrowhead; for CCAP). Double-labeling with anti-Pa-CCAP and Bm-CYC antibodies showed a similar co-localization pattern to that of CCAP-ir and CLK-ir (Fig. 3D-F, J-L, P-R); 2 small neurons between MI and ME (Fig. 3D-F arrows, Fig. 1C, for CCAP, arrow), in PFO (Fig. 3P-R, arrows; Fig.1B, arrow for CCAP), and in SM a large neuron lateral to NpMD (Fig. 3J- L arrows, Fig. 1 I, arrowhead; for CCAP).

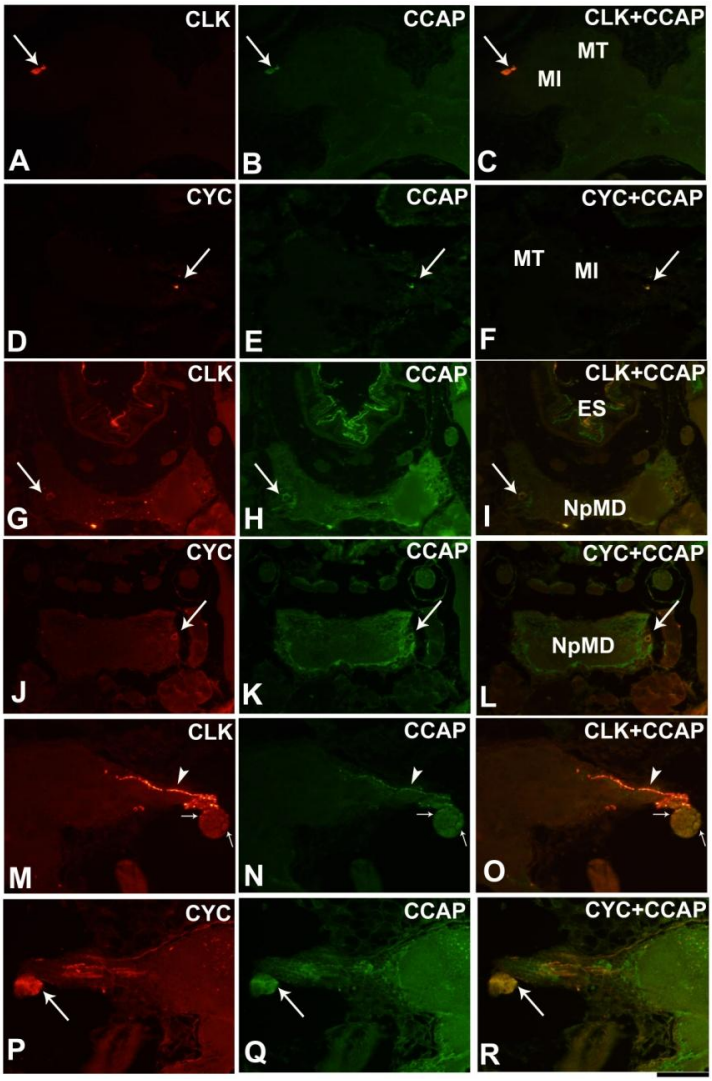

Figure 3:-Co-localization of CCAP-, CLK-, and CYC-ir in the cephalic ganglia of adult *A.vulgare*. (A, D, G, J, M, P) CLK-ir and CYC-ir were visualized by Alexa Fluor 555 (red fluorophore). (B, E, H, K, N, Q) CCAP-ir was visualized with Alexa Fluor 488 (green fluorophore). (C, F, I, L, O, R) pictures on the right show merged images. (A-C) a pair of CLK-ir (red) small cells and CCAP-ir (green) cells were identical in the OL (arrow). (D-F) a pair of CYC-ir (red) small cells and CCAP-ir (green) cells were identical in the OL (arrow). (G-I) a CLK-ir (red) large cell and CCAP-ir (green) cell were identical lateral to NpMD (SM) (arrow). (J-L) a CYC-ir (red) large cell and CCAP-ir (green) cell were identical lateral to NpMD (SM) (arrow) (M-O) CLK-ir (red) and CCAP-ir (green) in PFO (arrows), nerve fiber extend dorsally in OL (arrowhead) (P-R) CYC-ir (red) and CCAP-ir (green) were identical in the PFO (arrows). Scale bar 100 μm.

Co-localization of Crz-ir, CLK-ir, and CYC-ir in the cephalic ganglia of *A. vulgare*:-

Double-labeling with anti-Crz and *Bm*-CLK antibodies was conducted (Fig. 4A-C, G-I, M-O). The results revealed that Crz-ir and Clk-ir were colocalized in 2 small neurons between the MI and ME of the OL (Fig. 4A-C arrows, Fig.2C, arrowhead for Crz), and PFO showed both immunoreactivities (Fig. 4M-O, Fig.2B, arrowhead for Crz). A bilateral pair of large neurons in the SM adjacent to NpMD were similar in localization (Fig. 4G-I arrow, Fig 2L, arrowhead for Crz). Double-labeling with anti-Crz and *Bm*-CYC antibodies showed a similar co-localization pattern to that of Crz-ir and CLK-ir (Fig. 4D-F, J-L, P-R); 2 small neurons between MI and ME of the OL (Fig. 4D-F arrows, Fig. 2C, arrowhead for Crz), in PFO (Fig. 4 P-R, Fig.2B, arrowhead for Crz), and in SM pair of large neurons lateral to NpMD (Fig. 4J- L arrows, Fig. 2 L, arrowhead for Crz). Most likely these neurons contain these four proteins (CCAP, CRZ, CLK and CYC).

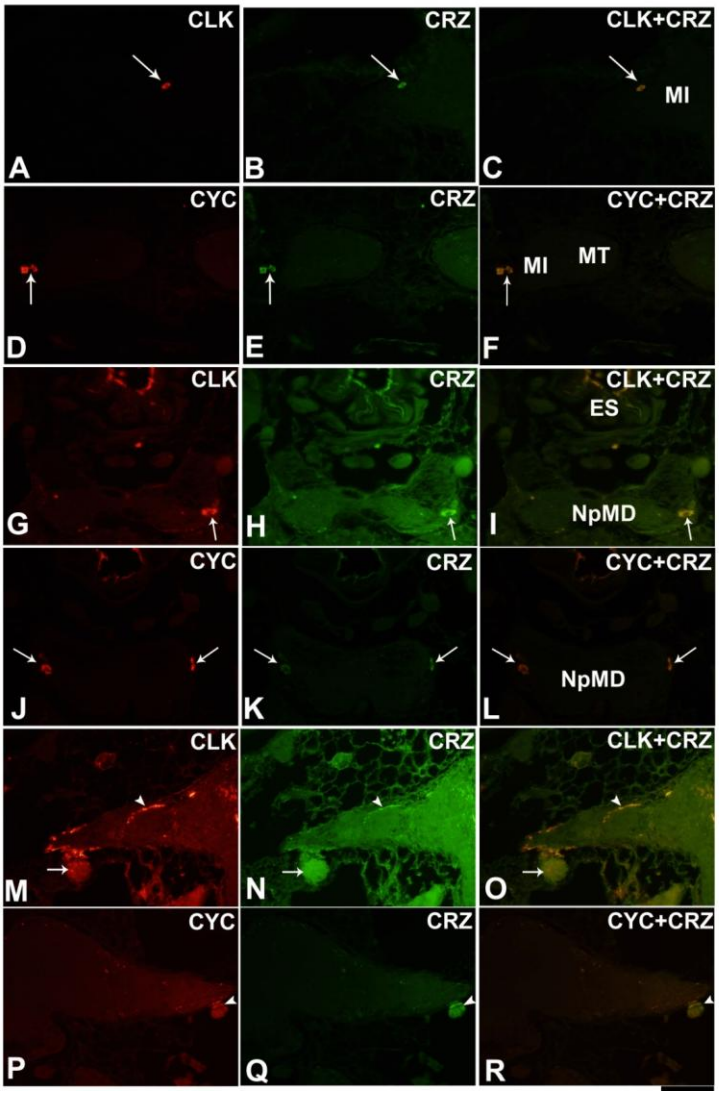

Figure 4:- Co-localization of Crz-, CLK-, and CYC-ir in the cephalic ganglia of adult *A.vulgare*. (A, D, G, J, M, P) CLK-ir and CYC-ir were visualized by Alexa Fluor 555 (red fluorophore). (B, E, H, K, N, Q) Crz-ir was visualized with Alexa Fluor 488 (green fluorophore). (C, F, I, L, O, R) pictures on the right show merged images. (A-C) CLK-ir (red) small cell and Crz-ir (green) cell were identical in the OL (arrow). (D-F) a pair of CYC-ir (red) small cells and Crz-ir (green) cells were identical in the OL (arrow). (G-I) a CLK-ir (red) large cell and Crz-ir (green) cell were identical lateral to NpMD (SM). (J-L) a CYC-ir (red) large cell and Crz-ir (green) cell were identical lateral to NpMD (SM) (arrow). (M-O) CLK-ir (red) and Crz-ir (green) in PFO (arrow), nerve fiber extend dorsally (arrow head) in OL. (P-R) CYC-ir (red) and Crz-ir (green) were identical in the PFO (arrow head). Scale bar 100 µm.

Discussion:-

Our previous investigation of *A.vulgare* has shown that antigens related to the clock proteins PER, CYC and CLK are confined to specific cells, some of which co-express PER-ir with either CYC-ir or CLK-ir and may actually contain all three antigens that represent the CPM of *A. vulgare* (Fouda, 2010). In *A. vulgare* similar distribution patterns of CCAP-ir and Crz-ir (Fig 1a,2a; Table 1) that are in regions in which the PER-ir, CYC-ir and CLK-ir cells are found (Fouda, 2010). Some of the cells expressing immunoreactivity for CCAP or Crz are actually either identical or lie in close proximity to the cells stained for a clock protein. Double-staining performed has established the co-localization of immunoreactivity for CCAP with CLK and CYC in two small neurons in OL, weak reactivity in PFO and large neuron in SM. The pattern is similar for both CYC and CLK with Crz. It seems that these antisera react with cells of identical morphology and position, suggesting that these neurons contain these proteins.

Stangier et al. (1988) studies revealed that CCAP-ir neurons within the thoracic ganglia of the crab were responsible for supplying CCAP to the peripheral neurohaemal pericardial organs (PCO) suggesting that PCO is a neurohormonal function. Furthermore, CCAP-ir was not confined to these ganglia but was also found in putative interneurons in all ganglia of the ventral nerve cord, suggesting CCAP to have neurotransmitter/neuromodulator functions (Dircksen and Keller, 1988).

In insects, CCAP secretion is under the control of an endocrine cascade (Truman, 2005) that may have more than one link to the circadian clock. The course of CCAP-ir and fibers often parallels the distribution pattern of processes containing the clock-related proteins in two cricket species (Shao et al., 2006; Sehadová et al., 2007). The finding of immuno-positive neurons recognized by the anti-CCAP antibody, suggests strongly the possibility of the existence of CCAP in the isopoda. CCAP-ir in the cephalic ganglia of isopoda is localized in the OL, PC, Al, TC and SM. CCAP has been found in the OL of the brain of many insects examined as *Baculum extradentatum*, *L. migratoria*, *D. nigrofasciatus* and *A. allardi*. (Dircksen et al., 1991; Lange and Patel, 2005; Sehadová et al., 2007). The optic lobe neurons that are associated with the medulla are speculated to be a part of a neuron group controlling circadian rhythmicity in insects (Wurden and Homberg, 1995). In *B. extradentatum* CCAP-ir neurons localized in the PC and deuterocerebrum (DC) and neuropil regions in the PC and TC, while in *D. nigrofasciatus* and *A. allardi* CCAP-ir occurs in PC cell, TC, SOG and frontal ganglion, this demonstrates the possible involvement of CCAP in the processing of a wide variety of behaviors (Lange and Patel, 2005; Sehadová et al., 2007). The immunopositive fiber in several regions of the brain and SOG is consistent with the notion that most insect neuropeptides act as neurotransmitters or modulators (Nässel, 2002; Sehadová et al., 2007).

Crz has not been isolated from the isopod chemically and no isopod gene encoding Crz has been described. However, structural conservation of Crz in various insects (Veenstra, 1989, 1991, 1994; Predel et al., 1999; Tawfik et al., 1999; Hua et al., 2000; Hansen et al., 2001; Roller et al., 2006; Verleyen et al., 2006) and Crz-ir somata in crayfish *Procambarus clarkia* (Porras et al., 2003) also identical corazonin from decapod crustaceans, the crabs *Cancer productus* and *C. borealis* (Li et al., 2003, Fu et al., 2005), the recent molecular data and the study of morphological characters suggest a very close relationship between Crustacea and Hexapoda (Tetraconata; see Richter, 2002).

The finding of Crz-ir strongly suggests the possibility of the existence of native corazonin or of a peptide of similar structure in the isopoda. Crz-ir in the cephalic ganglia of isopoda is confined to specific neurons in the OL, PFO, PC, TC and SM (Table 1). Corazonin is a hormonal neuropeptide in insects that is expressed in neurosecretory neurons of the pars lateralis of the PC and transported via nervi corporis cardiaci to the storage lobes of the corpora cardiaca (CC) (Predel et al., 2007). Crz-ir neurosecretory cells were located in the pars lateralis in *P. americana*, and the immunopositive material could be traced along the nervous corporis cardiaci-2 (NCC-2) to the storage lobes of the CC (Veenstra and Davis, 1993; Predel et al., 1994). A similar localization of Crz-ir material was found in the brain-retrocerebral complex of *L. maderae* and *P. americana* (Predel et al., 1994; Predel, 2001). The presence of Crz-ir perikarya in the fronto-lateral protocerebrum with projections to the ipsilateral CC was described in diverse insects (Roller et al., 2003; Hamanaka et al., 2004; Choi et al., 2005; Zavodska et al., 2008) and probably represents an ancient pattern. This agrees with results of isopoda which have positive neurons in OL that likes PC in insects. Also in the crayfish *P. clarkii* Crz-ir somata and fibers were identified in various regions of the eyestalk (MT, MI and ME) with the anti-corazonin antibody (Porras et al., 2003). Crz-ir somata in the SOG were described in *P. americana* (Veenstra and Davis, 1993), *D. nigrofasciatus* (Sehadová et al., 2007), the silverfish *Ctenolepisma lineata*, the bug *Pyrrhocoris apterus* (Roller et al., 2003) and in four termite species (Zavodska et al., 2008). On the

other hand, the SOG of nearly all insects contains Crz-ir fibers linking the brain with the ventral nerve cord. These fibers are missing in some crickets (Sehadová et al., 2007) and in four termite species (Zavodska et al., 2008).

The PFO of isopods have been shown to be homologous with the sinus gland of other malacostracans, therefore, having neurosecretory function (Walker, 1935; Amar, 1948, 1950; Gabe 1952, 1954; Oguro, 1960). Within the axon terminals of the PFO of *A. vulgare* three types of neurosecretory granules were identified (Taketomi and Tatsutoshi, 1986). Schmitaz (1989) reported that there are no nerve fibers or nuclei within the PFO of *A. vulgare*. PER-, CLK-, and CYC-ir detected and co-localized in PFO of *A. vulgare* suggesting the possibility that the clock proteins are secreted to the hemocoel (Fouda, 2010). In certain lepidopteran insects, similar mechanism of circadian rhythm generation has been proposed. For example, in *Antheraea pernyi*, there is a pronounced oscillation of PER-ir in the neurohemal organ (Sauman and Reppert, 1996). This agrees with our results as CCAP-ir, Crz-ir detected in PFO and co-localized with CYC and CLK. Taken together, this distribution is consistent with that the isopoda possess the CPM that associated with the OL and the SM (Fouda, 2010).

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