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IMMUNOLOCALIZATION OF TWO PUTATIVE PIGMENTOTROPINS AKH I AND α-MSH-LIKE IMMUNOREACTIVITIES IN THE BRAIN OF ROUGH WOODLOUSE, *PORCELLIO SCABER* AND PILL BUGS *ARMADILLIDIUM VULGARE* (CRUSTACEA, ISOPODA).

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Abstract

Antisera against putative pigmentotropic neuropeptides, Adipokinetic hormone I (AKH I) and α-Melanocyte stimulating hormone (α-MSH) reacted with small sets of neurons in the cephalic ganglia of the isopoda *Porcellio scaber* and *Armadillidium vulgare*. The distributions of immunoreactivities resembled in the two species. AKH I-like immunoreactivity (AKH I-ir) occurs in both species. In *P scaber* 5 cells occurred in the optic lobe (OL) (3 small near Lamina ganglionaris, LG and 2 large dorsally) while in *A.vulgare* only one small cell near LG. The accessory lobe (AL) and subesophageal mass (SM) have the same pattern in both species. There are 2 cells in AL and 6 cells in SM (in the cells of mandibular ganglion,CMD). α-MSH immunoreactivity (α-MSH-ir) were located in OL, and SM in both species and in AL of *A.vulgare*. In *P. scaber* two large perikarya were located dorsally, one small ventrally, while in *A.vulgare* 4 large dorsally, one small ventrally and strong reactivity in pseudo-frontal organ (PFO). In AL, 3 immunopositive cells were observed. In SM 6 cells (4 in CMD, 2 in the cells of maxillulary ganglion, CML) in *P scaber* and 8 cells (6 in CMD, 2 in CML) were observed in *A. vulgare*. These results suggest the possible existence of AKH-like and α-MSH-like peptides in isopoda. No differences were detected between males and females. The projections of immunoreactive fibers were traced to several brain regions, the somatogastric nervous system and the neurohaemal organs, which suggest multiple functions of these peptides.

Key words: Immunohistochemistry; Adipokinetic hormone; α-Melanocyte stimulating hormone, *Porcellio scaber* , *Armadillidium vulgare,* Isopoda.

Introduction

Some crustaceans exhibit reversible and rapid changes in integumental and eye pigments to adapt to background light conditions. The color changes result from pigment movements within epithelial chromophore cells by concentrating or dispersing the pigment granules. The chromatophores are classified under four types: erythrophores, xanthophores, leukophores, and melanophores. (Rao, 1985; Rao and Riehm, 1988 ; Yang *et al.,* 1999; Ohira *et al.*, 2006). The eyestalks, that contain neurosecretory centers and neurohemal organs (sinus glands) have long been known as the major source of diverse pigmentary-effector peptide hormones (Fingerman, 1963; Rao,1985). *β*-PDH and RPCH are the major peptides secreted from the X-organ/sinus gland neuroendocrine complex in the eyestalk of crustaceans, with the main action in pigment migration. *β*-PDH promotes pigment dispersion in all types of chromatophores and RPCH promotes pigment aggregation in erythrophores in brachyuran and isopod species, and in leucophores, melanophores and xantophores in other species (Josefsson,1975; Skorkowski and Biegniewska,1981; Yang *et al.*,1999).

Adipokinetic hormones (AKHs) in insects were found to be structurally related to RPCH (Mordue and Stone, 1976; Gade, 1990, 1991) and a large family of AKH/RPCH peptides has been documented that cause pigment concentration in erythrophores (Rao, 1985; Rao and Riehm, 1988). Insect AKH has a primary structure varying from 8 to 10 amino acid residues, depending on the species. The most commonly identified function is the control of lipid and carbohydrate metabolism (Gade and Auerswald, 2003; Gade *et al.*, 1997; Van der Horst *et al.*, 2001). AKHs also produce behavioral effects (Kodrik *et al.*, 2000; Lee and Park, 2004). AKH I (Stone *et al.*, 1976) has the first four and the last amino acids in common with RPCH. Up to two other distinct sequences have been found in a single species, for example, AKH II and AKH III from *Locusta migratoria.* AKHs are unusual neuropeptides for several reasons, first; they are generally present in large quantities in the corpora cardiaca (CC) (Gade *et al.*, 1997). Second, the structures of the peptides identified from the various insect species vary, clearly much more than other similar sized insect neuropeptides or the closely related crustacean RPCH (Yamashiro *et al.*, 1984). Third, some insect species have two or three different AKHs whose functions are likely to be different, since they have slightly different physiological effects (Gade *et al.*, 1994, 1997; Park and Keeley, 1998). Crustaceans contain only one member of the AKH/RPCH family, i.e., Panbo-RPCH, which was the first member of this family fully chemically characterized from a prawn (Fernlund and Josefsson, 1972). The same peptide was found in diverse species, ranging from prawns to crabs, crayfish, lobsters and spiny lobsters representing different infraorders and superfamilies (Gade, 2009), while in insects, the primary structures of 47 different forms of the family have been identified. (Gade *et al.*, 1997; Lee *et al.*, 2000; Gade, 2009). Of particular interest is that biological cross-reactivity has been demonstrated between members of these two groups. Thus, AKH may induce pigment aggregation in crustaceans and RPCH elicits adipokinetic effects in insects (Mordue and Stone, 1977). These structural and functional similarities between RPCH and AKH led to the notion of an RPCH/AKH family (Gade *et al.*, 1997). Because of their structural relationship, RPCH and AKHs mimics each other in cross-tests and elicit hyperlipemia in locusts, hyperglycemia in cockroaches and chromatophore pigment concentration in shrimp (Rao, 1985 and Fingerman, 1988).

In vertebrates α-MSH is the most important hormone in pigment dispersion (Eberle, 1988; Filadelfi and Castrucci, 1994; Zhu and Thomas, 1997). α-MSH has 13 amino acid residues, belongs to a family of peptides derived from a precursor molecule called pro-opiomelanocortin (POMC) (Nakanishi *et al.*, 1979). It is highly conserved among vertebrate species and is included in the amino-terminal sequence of ACTH (Kawauchi *et al.*, 1984). α-MSH is synthesized in the pars intermedia (PI) of the adenohypophysis (ADH), probably acting as a neurotransmitter or neuromodulator (Vallarino *et al.*, 1988, 1989). Melanocyte-stimulating hormone

(MSH) was extracted and purified from ADH of mammals, it is released from the hypothalamus. It binds to the receptors on melanocytes and melanoma cells to affect the distribution of melanin. The importance of this action is mainly protective to color change in lower vertebrates. In higher vertebrates MSH has taken a wide variety of other functions, it is classified into α, ß and γ MSH. The melanin-concentrating hormone (MCH) is the antagonist of α-MSH, evoking pigment aggregation (Fujii and Oshima, 1994). Colocalization of MCH and α-MSH has been reported in several vertebrate species: rat (Naito *et al.*,1986; Pelletier *et al.*, 1987), frog, *Rana ridibunda* (Andersen *et al.*, 1987), and dogfish, *Scyliorhinus canicula* (Vallarino *et al.*, 1989). Colocalization of α-MSH and MCH in the same neurons could not be demonstrated, but the relationship between MCH and α-MSH neurons in similar brain nuclei and the close association between MCH fibers in the neurohypophysis (NH) and the α-MSH pituitary cells suggest that these two peptides may exert a coordinated hormonal activity during background color adaptation (Pandolfi *et al.*, 2003). In invertebrates few reports demonstrated MSH-ir (Martin *et al.*, 1980, Marchand and Dubois, 1982; Van Deijnen *et al.*, 1985; Dhainaut-Courtois *et al.*, 1985) and in insects (Veenstra, 1984; Hansen *et al.*, 1986; Schoofs *et al.*, 1987).

Much information about pigmentotropins is available in many crustacean species and insects but not in isopoda. In this study, antibodies against AKH I and α- MSH were used to localize AKH I and α-MSH-ir in the cephalic ganglia of two isopod species *P. scaber* and *A. vulgare*.

Material and Methods

Animals and sample preparation

Adults of two isopod species *P. scaber* and *A. vulgare* were collected on Rokkodai campus of Kobe University, Japan (34^o 73 N and 135^o 23 E) and kept at 25^{o}C under LD 12:12 for at least 7 days before they were sacrificed in the middle of the photophase (between 4 and 8 h after lights-on).

Immunohistochemistry

Immunohistochemistry was performed as described by Shao *et al.* (2006). Whole heads were separated from anesthetized animals in sterile saline and fixed overnight at 4°C in Bouin’s solution (15 vol. picric acid, 5 vol. formalin, 1 vol. acetic acid). Standard techniques were employed to prepare tissue sections (8 μm) in paraplast. Following deparaffinization, sections were washed in 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 and incubated with the respective primary antibody overnight at 4°C in a humidified chamber. The antisera were diluted in TBS-T as follows: anti-AKH I antiserum 1:200 and anti α-MSH antiserum, 1:100. Further processing was done at rt. Bound antibody was detected with the rabbit IgG-Vectastain Elite ABC kit (Vector Laboratories, Burlingame, Calif.). The activity of the horseradish peroxidase (HRP) conjugated to the 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). Stained sections were mounted in Bioleit medium (Kouken Rika, Osaka, Japan) and visualized under a BX50F4 microscope (Olympus, Tokyo, Japan) equipped with Nomarski contrast, epifluorescence optics and a charge-coupled device camera.

Specificity to the primary antibody

Rabbit polyclonal antibody AKH I (9 amino acid residues) (Gene Med, Texas) and rabbit polyclonal anti α-MSH (13 amino acid residues) provided by Dr.N. Yanaihara (former professor of Shizuoka Prefectural University) were used. For control, the 2 antibodies were replaced with normal serum where no cross reactivities of the primary or secondary antibodies were observed. The specificity of the 2 antibodies were confirmed with preadsorption test where each antigen (1 ng/ml) was diluted together with its antibody (anti AKH I-antibody 1:200 and anti α-MSH antibody, 1:100) in TBS buffer with a ratio of 1:5 and incubated overnight at 4 C. The preadsorbed serum was used in place of the primary antibody in the usual immunohistochemical protocol. As a result, the immunoreactivity entirely disappeared after antibody preadsorbed with the antigen in the two antibodies.

Results

AKH immunoreactivity (AKH-ir)

Fig.1 shows abbreviations of morphological land mark in the isopod brain. The AKH-ir appeared in *P. scaber* and *A.vulgare*. Some similarities were noticed between the two species. AKH-ir occured in the OL, PC and SM, while no reactivity was observed in the CB, DC and TC in both species (Fig. 2 a,3 a). At the base of OL three positive neurons and some varicose fibers close to LG were observed in *P. scaber* (Fig. 2 b) while in *A.vulgare* only one somata was detected at this part (Fig. 3b). Two large strongly stained perikarya were seen in the doral side of the OL of *P. scaber* (Fig. 2 c,d;arrow head) with little arborization centrally near MT (Fig. 2d, arrow) but no immunopositive perikarya seen in the PC of both *P. scaber* and *A.vulgare*. The pattern of staining in the central brain was similar in *P. scaber* and *A.vulgare*, both species harbouring 2 positive neurons in the AL (Fig. 2 e,f and 3 c,d) and no immunoreactivity seen in the CB, DC and TC. In the SM AKH-ir was detected in both species with slight difference in distribution. In *P. scaber* the CMD had two pairs of large perikarya centrally located (Fig. 2 g,i; arrow head) while on the edge one large strongly stained perikaryon (Fig.2 h,i; arrow). A projection seemed to connect between the central cells in CMD in the dorsal side (Fig. 2j). Varicose fibers were detected on the edges near connection between SM and CEC (Fig. 2k). while in *A.vulgare* CMD with one large finely stained perikaryon centrally located (Fig. 3e,f). A neurite was detected centrally (Fig. 3g) and two large strongly stained perikarya on the edges (Fig. 3 g,h; arrow), strongly stained varicose fibers at connection between SM and CEC observed (Fig. 3 i). Varicose fibers occurred within the CML in both species (Fig. 2L,3 j).

α-MSH immunoreactivity (α-MSH-ir):

α-MSH-ir occurred exclusively in the OL and SM in *P. scaber*. *A.vulgare* harboured positive neurons in the AL also, while DC and TC had no reactivity (Fig.4a,5a). Two large perikarya were detected in dorsal part of OL in *P. scaber* (Fig. 4b,c). Moderately stained small neurons were observed in the ventral part of OL with arborization extending ventrally towards the PC (Fig. 4 d,e). The AL was devoid of staining, while in the dorsal part of OL of *A.vulgare* 4 large immunoreactive perikarya were observed (Fig. 5b,c,d,e) and one small strongly positive and ventrally located (Fig. 5f). The accessory lobe showed strong reactivity, two large perikarya (Fig. 5 g,h) and one small (Fig. 5 i). α-MSH-ir was observed in PFO of *A.vulgare* (Fig. 5 j) but not at all in PFO of *P. scaber*. In the SM, α-MSH-ir was detected in both species with variation in number; in *P. scaber* two cells, one large and one small near the edge of CMD (Fig. 4f,g), while CML had a pair of small cells (arrow) and varicosity ventrally,arrow head (Fig. 4 h). In *A.vulgare*, a pair of large neurons centrally located (Fig. 5 k) and two pairs of small cells were observed on the edge (Fig. 5 l,m). CML had a pair of large strongly stained neurons (Fig. 5 n), arborization seen centrally (Fig. 5 o). No difference was recorded between males and females in examined antibodies.

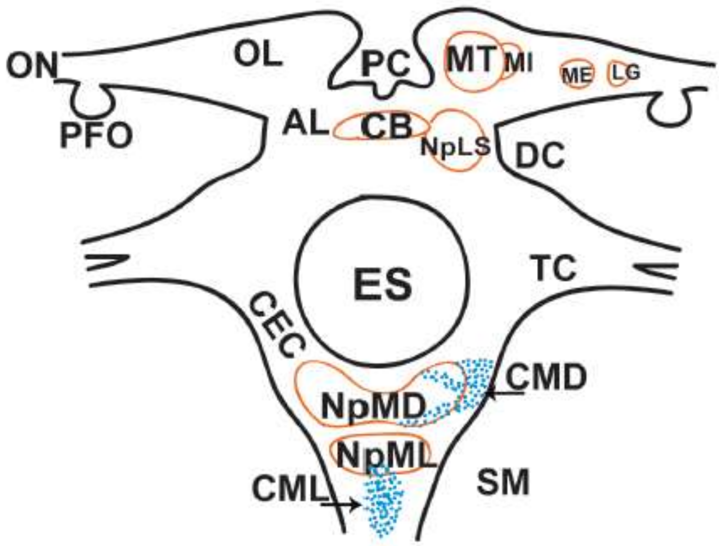

Fig.1 A schematic diagram illustrating the cephalic neural complex of Isopoda with abbreviations used in the photographs (ON, optic nerve; OL, optic lobe; PC, protocerebrum; MT, medulla terminalis; MI, medulla interna; ME, medulla externa; LG, lamina ganglionaris; PFO, pseudo-frontal organ; CB, central body; AL, accessory lobe; DC, deutocerebrum; TC, tritocerebrum; ES, esophagus; CEC, circumesophageal connective; SM, subesophageal mass; NpLS, neuropile of accessory lobe; NpMD, neuropile of mandible; CMD, cells of mandibular ganglion; NpML, neuropile of maxillule; CML, cells of maxillulary ganglion).

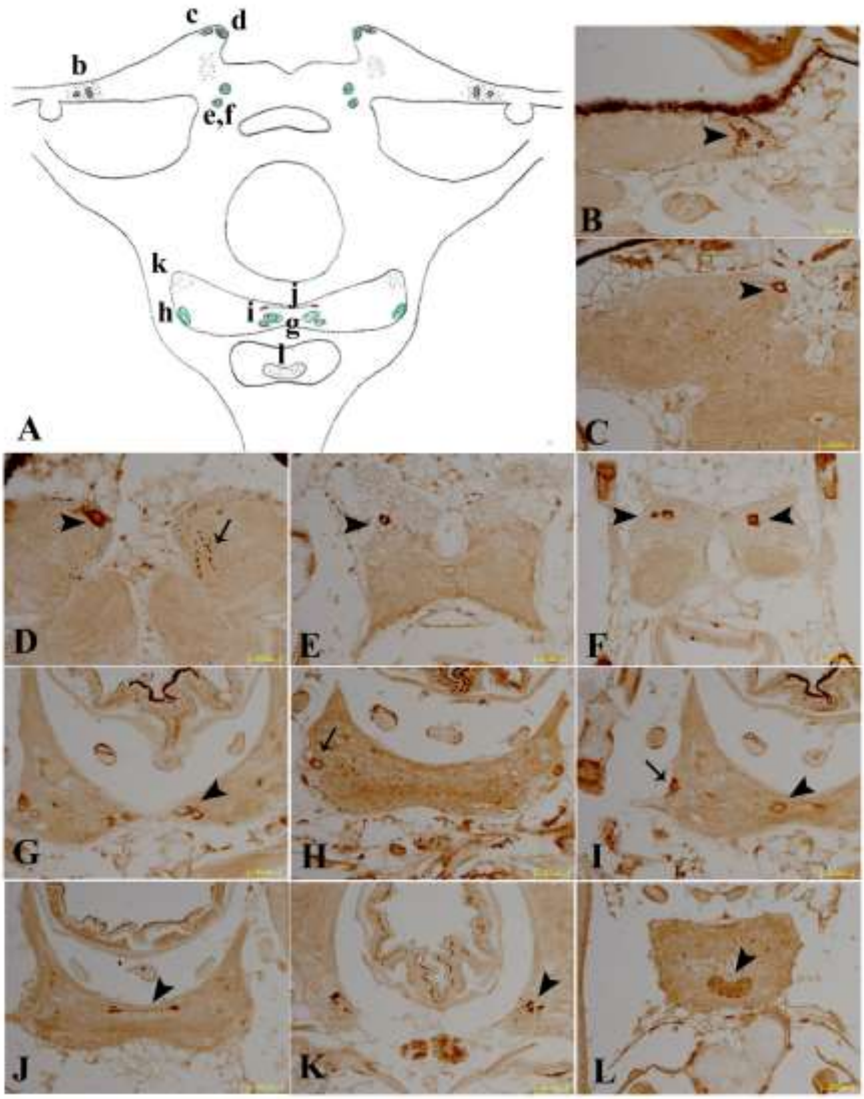

Fig. 2 AKH immunoreactivity (AKH-ir) in the cephalic ganglia of adult *P. scaber* a Representation of the numbers and topography of AKH -ir cells and the pathways of their projections (a–l positions of the respective micrographs in this figure). b, Positive neurons at LG. c-d, Large strongly stained perikarya in dorsal side of OL(arrow head). d, Arborization near MT (arrow). e-f, Positive neurons in AL. g and i, Large perikarya centrally located in NPMD (arrow head). h-i, Large strongly stained perikarya in edge of NPMD (arrow). j, Immunopositive projection like centrally near NPMD. k, Varicose fibers on the edge between SM and CEC. l, Varicose fibers near NPML. Scale bar 50 µm.

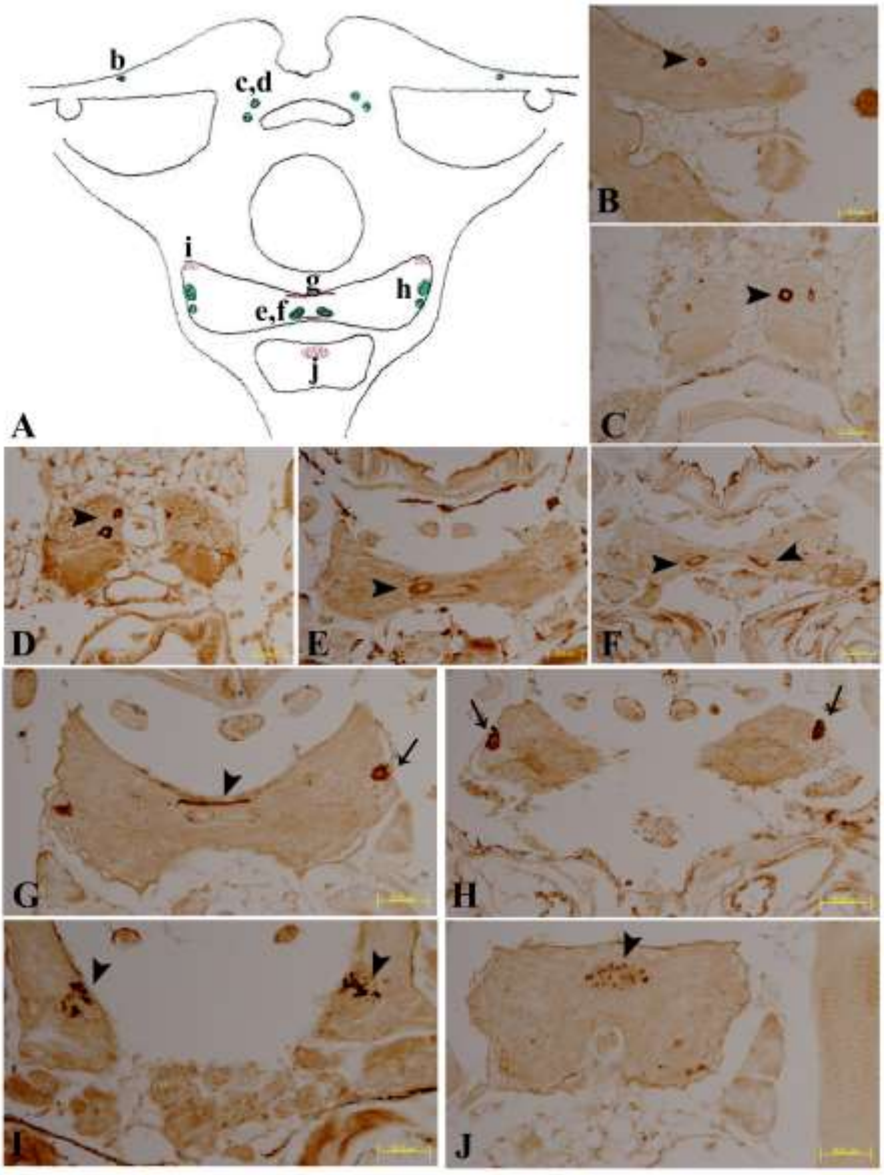

Fig. 3 AKH-ir in the cephalic ganglia of adult *A.vulgare* a Representation of the numbers and topography of AKH -ir cells and the pathways of their projections (a–j positions of the respective micrographs in this figure). b, Positive somata at LG. c-d, Positive neurons in accessory lobe. e-f, Large perikarya centrally near NPMD, g, Immunopositive neurite centrally near NPMD. g-h, Strongly stained perikarya near the edge of NPMD (arrow). i, Varicose fibers at connection between SM and CEC. j, Varicose fibers close to NPML. Scale bar 50 µm.

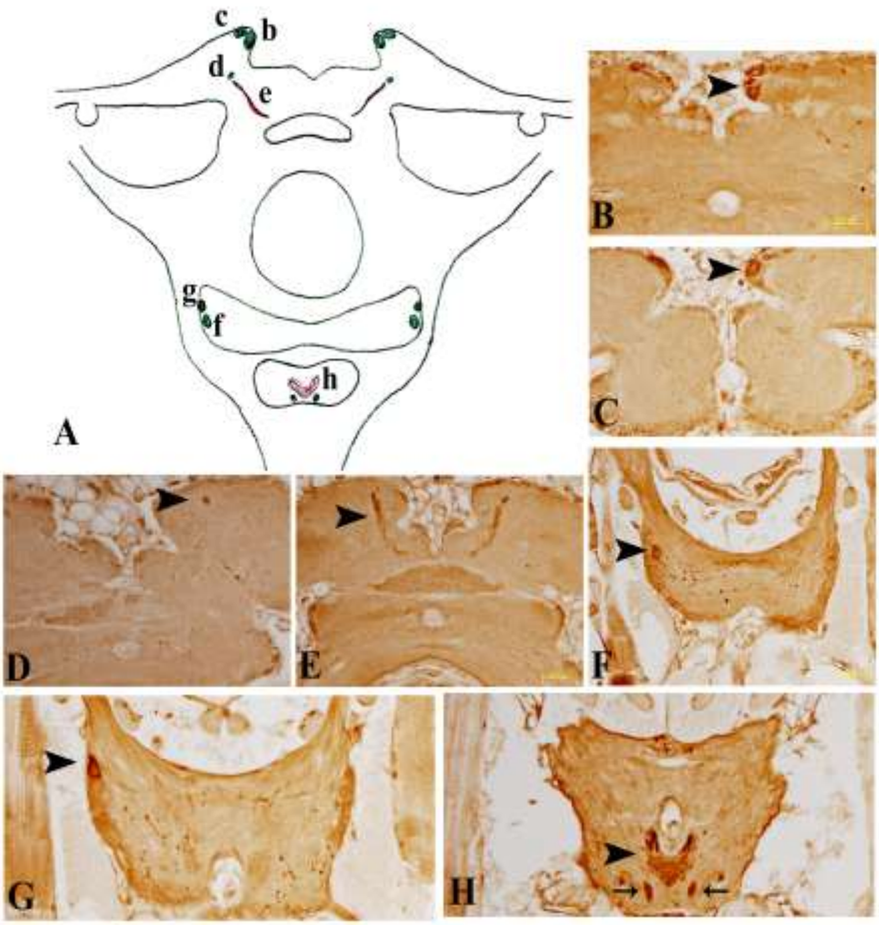

Fig. 4 α-MSH immunoreactivity (α-MSH-ir) in the cephalic ganglia of adult *P. scaber* a, Representation of the numbers and topography of α-MSH -ir cells and the pathways of their projections (a–h positions of the respective micrographs in this figure). b-c, Large positive neurons in dorsal part of OL. d-e, Moderately stained small neuron ventrally in OL, arborization towards PC. f-g, Positive neurons near edge near NPMD. h, Pair of positive cells (arrow) and varicosity (arrow head) near NPML. Scale bar 50 µm.

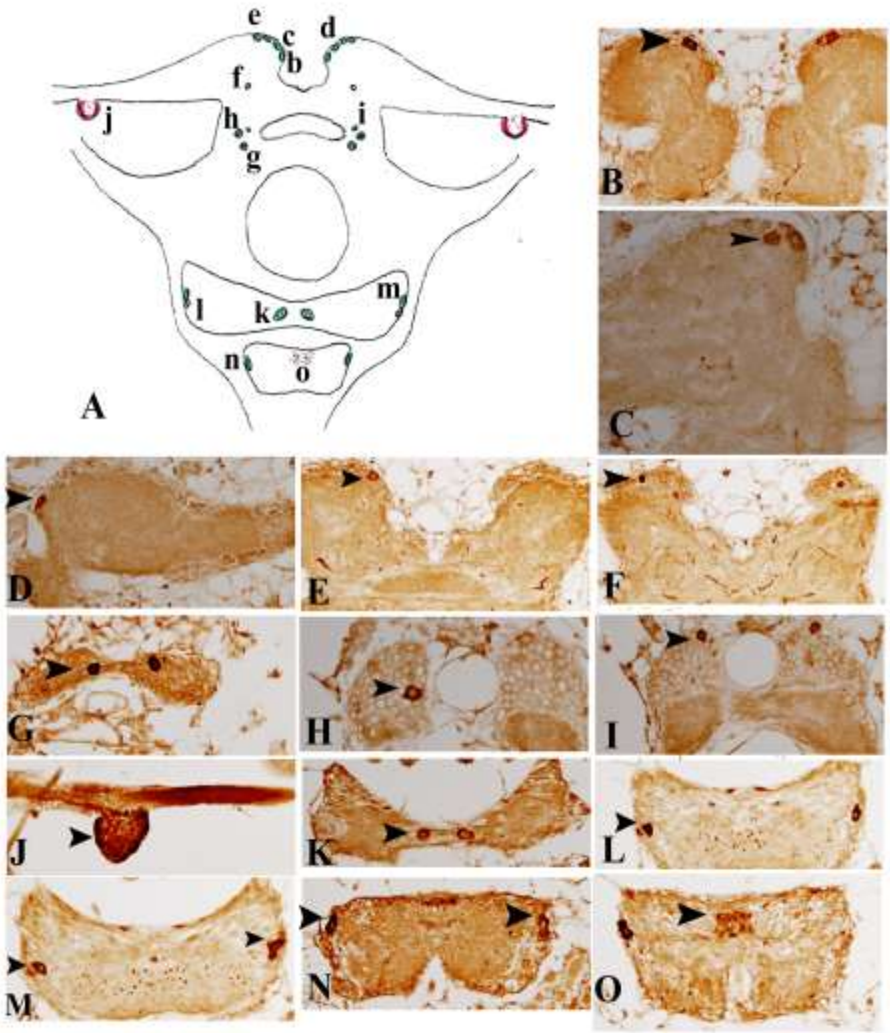

Fig. 5 α-MSH-ir in the cephalic ganglia of adult *A.vulgare* a, Representation of the numbers and topography of α-MSH -ir cells and the pathways of their projections (a–o positions of the respective micrographs in this figure). b – e, Large immunopoitive neuron in dorsal part of OL. f, Small strongly stained neuron in OL ventrally located. g-h, Large strongly reactive neurons in AL. i, Small positive neuron in the AL. j, Strong reactivity in PFO. k, Large neurons centrally near NPMD. l-m, Small positive cells in edge of NPMD. n, Large strongly stained neurons near NPML. o, Arborization centrally near NPML. Scale bar 50 µm.

Discussion

The distribution of AKH-ir

The X organ-sinus gland system in the crustacean eyestalk secretes various neuropeptides, Their chemical structure suggests the existence of at least three families (Keller, 1992; Garfias *et al.*, 1995; Garcıa and Arechiga, 1998). The CC is the major organ of the insect neuroendocrine system that store neurohormones and release them into the circulation. AKH I and AKH II are synthesized by neurosecretory cells (NSC) of the CC in the locust, *Schistoeerca gregaria*.(Siegert *et al.*, 1985; O'Shea and Rayne, 1992). In the locust the NSC cells intrinsic to the CC are clustered together in the so-called glandular lobes. The neurosecretory cells of the brain send axons to the CC through two pairs of nerves and these axons arborize in separate lobes called the storage lobes. The AKH are located in and synthesized by the intrinsic neurosecretory cells of the glandular lobes (Goldsworthy *et al.*, 1972; Hekimi and O'Shea, 1987). Several lines of evidence show that individual neurosecretory cells of the CC make both AKH I and AKH II. This can be seen clearly by immunocytochemical labelling of the glandular lobe using antibodies with high specificity for AKH I and AKH II (Hekimi and O'Shea, 1989).

The present data showed that AKH-ir in the cells in the OL, AL. and SM in both species, in *A. vulgare* only one cell was detected in the LG while in *P. Scaber* 3 small cells were detected in LG and 2 big cells in the dorsal side of OL. In the AL both species harboured a pair of positive neurons. In the SM, NPMD had 6 cells, 4 centrally and 2 near the edge in *P. scaber* while in *A.vulgare* a pair of large perikarya was centrally located and 2 pairs near the edge, in NPML only varicose fibers in the both species.

Many insects showed AKH-ir in CC as in *Carausius morosus* which showed AKH-I ir in cell bodies of glandular part of CC, *Sarcophaga bullata* showed cell bodies and nerve fibers of AKH-I in CC and corpora allata (CA) and ir-neurons in parslateralis (Clottens *et al.*, 1989), in *D.melanogaster* AKH-ir was localized in CC (Isabel *et al.*, 2005), in *P. americana* AKH-ir was detected in several neuron types seen in the abdominal ganglia, terminal ganglia as well as axons running along the mid gut (Schaffer, 1986). While in hard tick *Rhipicephalus appendiculatus* antibody to Manduca AKH failed to react or showed inconsistent staining with any described cells or structures (Simo *et al.*, 2009)**.**

The distribution of α-MSH-ir

In Amphibia the pituitary peptide hormone, melanophore-stimulating hormone accounts for the darkening of the skin in response to a black background. Since then, the biological effect of MSH on skin pigmentation was studied extensively in vertebrates (Bunel *et al.*, 1992; Vallarino *et al.*, 1998; Jegou *et al.*, 1993). Colocalization of α-MSH and MCH has been reported in the dorsolateral hypothalamic region of the rat (Naito *et al.*, 1986; Fellman *et al.*, 1987), in the preoptic nucleus of the frog, *Rana ridibunda* (Andersen *et al.*, 1987), and in the nucleus sacci vasculosi in the brain of the dogfish, *Scyliorhinus canicula* (Vallarino *et al.*, 1989) and lungfish, *Protopterus annectens* (Vallarino *et al.*, 1998) and cichlid fish *Cichlasoma dimerus* (Pandolfi *et al.*, 2003). In the brain of invertebrates only a few reports demonstrate MSH-like immunoreactivity. In cephalopods (Martin *et al.*, 1980) and crustaceans (Van Deijnen *et al.*, 1985) an α-MSH has been detected. ß-MSH-ir is present in gastropods (Marchand and Dubois, 1982) and in annelids (Dhainaut-Courtois *et al.*, 1985). In insects, α-MSH-resembling peptides have been shown in the subesophageal ganglion (SOG) of *Locusta migratoria* and the Colorado potato beetle *Leptinotarsa decemlineata* (Veenstra, 1984) and in the CC-CA complex of *Leucophaea maderae* (Hansen *et al.*, 1986). α-MSH and ß-MSH-ir cells and nerve fibres were demonstrated within the nervous system of adults and larvae of *L. migratoria* and 3, 5 and 8 day old adult *Sarcophaga bullata* (Schoofs *et al.*, 1987). Injecting α-MSH into albino locusts causes its darkening (Tawfik *et al.*, 1999).

The present data showed that α-MSH-ir occurs in both species. 4 large neurons dorsally in the OL of *A. vulgare* but only two in *P.scaber*. Both specie harbour a small neuron ventrally. In the AL 3 α-MSH-ir neurons were detected in *A.vulgare* including PFO. This agrees with other crustaceans. In the crayfish *Astacus leptodactylus* the OL and sinus gland showed α-MSH-ir. (Van Deijnen *et al.*, 1985). The SM showed reactivity in both species, NPMD harbouring 6 cells in *A.vulgare* and *P. scaber* only 4. NPML had 2 cells in both species, The reactivity in the OL and SM were detected. *Sarcophaga bullata* α-MSH-ir cells were located in lateralis at the basis of the OL, in the SOG and at sides of the oesophageal foramen, and in *L. migratoria* α-MSH-ir cells were located in PC, SOG, ventral nerve cord and occasionally weakly stained fibers were observed in the CC (Schoofs *et al.*, 1987). This confirms the possibility of existence of α-MSH in isopoda.

The structure of RPCH is closely related to AKH. RPCH has a functional role opposite to that of PDH (Garfias *et al.*, 1995; Rao, 1985; Gaus *et al.*, 1990). In our results some similarity was observed between PDF-ir (unpublished data) and AKH-ir in some positions (LG, dorsal part of OL and NPMD) in *P. scaber* and in NPMD in *A. vulgare* (Fouda *et al.*, 2010) and the other cells are different in position. More information is needed to understand their role and the variation in number and the location of these neuropeptides in the two species.

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