ZOOLOGICAL SCIENCE 8: 905–913 (1991)

Distribution of Methionine-Enkephalin-Like and FMRFamide-Like Immunoreactivities in the Central Nervous System (Including Dorsal Bodies) of the Snail *Helix aspersa* Müller

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ABSTRACT—We investigated the immunocyto-localization of methonine-enkephalin and of FMRFamide in the central nervous system and in the dorsal bodies of adult snails *Helix aspersa*.

The results summarized on maps demonstrate that:

1) Methionine-enkephalin- and FMRFamide-like substances are detected in many neurons of all ganglia. They are never colocalized.

2) The areas of supra- and subesophageal methionine-enkephalin positive dorsal bodies are innervated by a network of FMRFamide positive fibers, which contain immunogold positive granules and establish close contacts with the dorsal-body cells.

The extensive distribution of methionine-enkephalin- and FMRFamide fibers and cells of various sizes suggests that these peptides play different functions in the nervous system. A special attention is paid to the control of the dorsal bodies according to previous functional results.

INTRODUCTION

In *Helix aspersa,* as in other Stylommatophora, the central nervous system (CNS) is built up of 9 ganglia surrounded by a connective tissue sheath, the perineurium, which contains non-nervous endocrine cells, the so-called dorsal bodies.

The neurosecretory cells of the circumesophageal ganglia have been studied by classical methods (chrome-haematoxylin, paraldehyde-fuchsin) [1] and by the alcian blue-alcian yellow technic [2, 3]. The use of immunocytochemistry has recently given complementary information and may help to define neuronal sub-populations. Moreover the supra- and sub-esophageal localization of the dorsal body cells has been made more precise by their immunoreactivity with antibodies to methionine-enkephalin [4]. However it is well established that the dorsal bodies of *Helix aspersa* are endocrine organs which control vitellogenesis and the synthetic activity of the female accessory organs [5–8], but the hormones of the dorsal bodies have not yet been identified. They are under inhibitory nervous control, as demonstrated by *in vivo* and *in vitro* experiments [6, 8–10], and their innervation probably originates from the Cerebral Green Cells (CeGC).

There have been some papers reporting the existence of methionine-enkephalin [11] and FMRFamide [12, 13] immunoreactivities in *Helix* CNS. The present paper compares the distribution of substances related to these both peptides in the CNS of *Helix aspersa.* It points out the abundance of neurons with immunoreactivity like that of either peptide, the abundance of axons innervating the dorsal body areas and their immunoreactivity mainly to anti-FMRFamide.

Accetpted June 20, 1991
Received May 8, 1991

MATERIALS AND METHODS

Animals

Adult snails were bred under controlled artificial conditions (long-day light cycles 18 hr L-6 hr D, at 20°C and 90% relative humidity) and fed *ad libi-*

tum in the Centre Universitaire d'Héliciculture de Besançon.

All of the animals used in these investigations (10) were sexually active and were sacrificed just before mating.

Methods

The circumesophageal nerve rings were excised and immediately immersed in Bouin-Hollande fixative (+10% $HgCl_2$) for 24 hours. The 6 μm paraffin sections were processed for immunocytochemistry, as described previously, by the three step method [4, 14–16] involving the peroxidase-anti-peroxidase method [17]. Pieces of connective tissue containing dorsal body cells were removed dorso-frontally to cerebral ganglia, fixed for 2 hr in a mixture of 2% glutaraldehyde, 0.1 M sodium cacodylate, 0.17 M NaCl (1v/1v/1v), washed overnight in 0.1 M buffer and embedded in epon-araldite. The ultrathin sections were treated by the immunoglod method as reported earlier [18].

The primary antibodies were raised in rabbits to the carboxy terminals of synthetic methionine-enkephalin and FMRFamide and were used in final dilutions of respectively 1/3000 and 1/8000. These antibodies were prepared and maintained in our laboratory [14, 19]. The two antibodies do not cross-react and are specific to the antigen they were directed to [20]. In any case, the structural similarities between the two peptides led us to compare adjacent sections in order to determine whether peptides related to, or similar to, methionine-enkephalin and FMRFamide are colocalized.

Two maps of the intra- and extra-ganglionary localization of immunoreactive structures were drawn by projecting each of them on to the same sketch. Particular attention was given to the CeGC [3] in order to establish the origin of the nervous fibers innervating different areas in the vicinity of the ganglia.

RESULTS

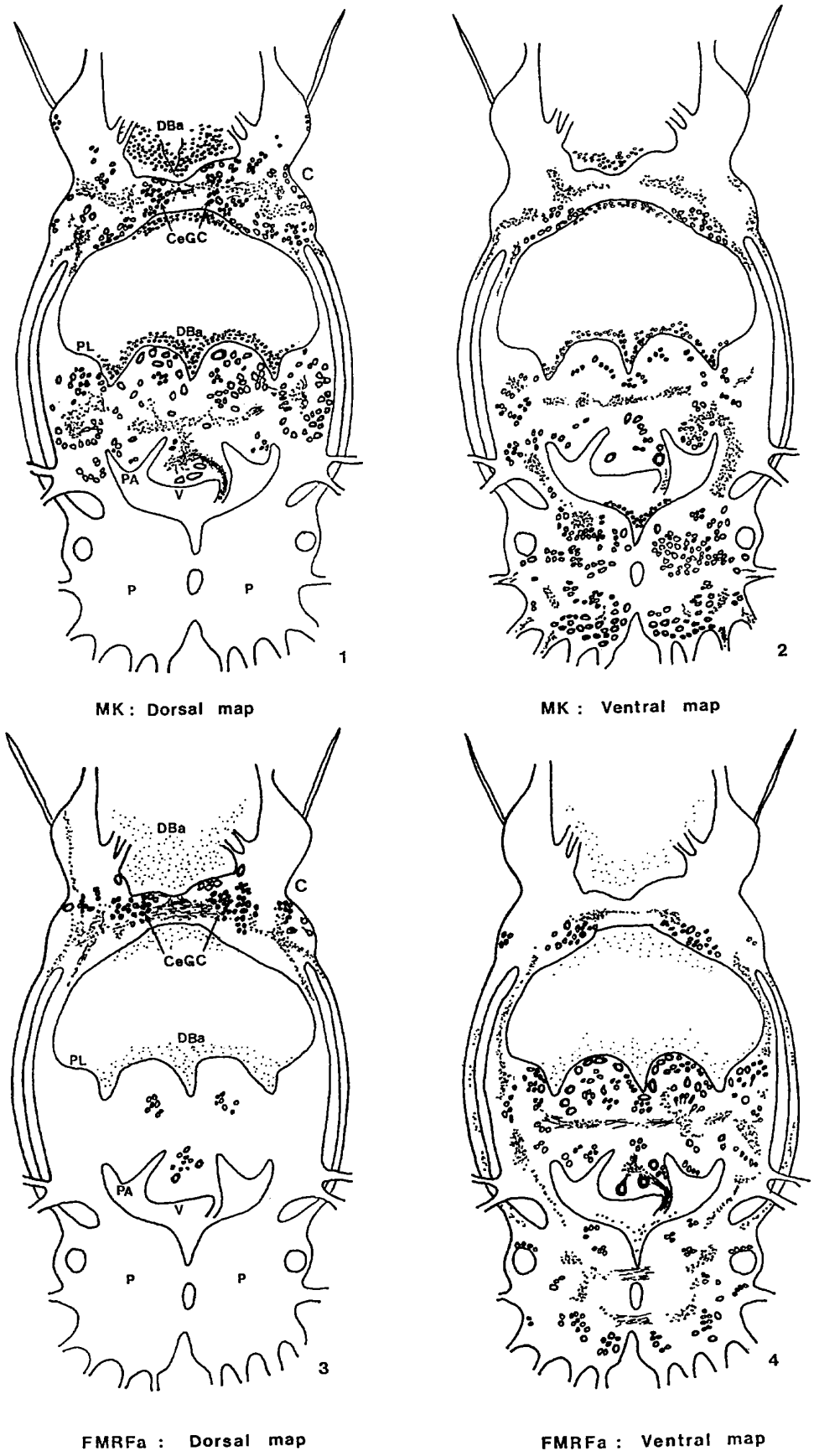
Intra-ganglionic immunoreactive structures

Observations of serial sections of entire nervous systems have shown a lot of immunoreactive perikarya and fibers in the different ganglia, in the neuropiles and in the different commissures and connectives.

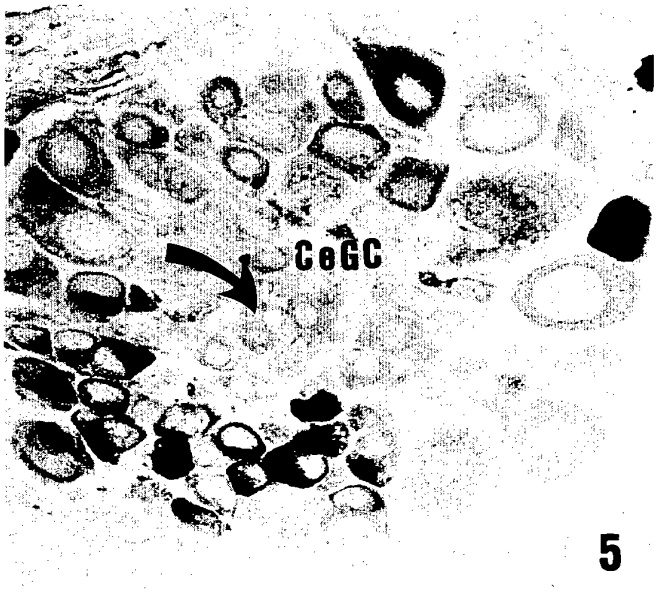
Methionine-enkephalin immunoreactivity: Immunoreactive cells of different sizes were observed in all ganglia (Figs. 1, 2). In the cerebral ganglia, they are small. Most of them are located on the dorsal surface. A few cells of 10 μm in diameter are scattered in the procerebrum, especially in the medial and latero-dorsal regions. In the metacerebrum, 25–30 μm positive cells are dispersed dorsally or located along the lateral margins of the ganglia. Smaller cells (15–20 μm) extend along these margins or are concentrated at the posterior region of the meta- and mesocerebrum. As for the so-called CeGC, most of them are methionine-enkephalin positive (Fig. 5), except for some which form the lobes on the anterior part of mesocerebrum.

In the subesophageal ganglia, immunoreactive cells are interspersed with negative cells of similar size. Concerning the immunoreactive cells, the pleural ganglia exhibit cells of 10 to 15 μm in diameter at the fronto-dorsal region and 25–30 μm cells in a more posterior position. The parietal ganglia contain big cells, one or two cells of 90–95 μm and cells of 50 to 60 μm at the anterior part, cells of 40 μm posteriorely. The visceral ganglion (Fig. 7) contains cells of various sizes among which some big positive cells (up to 100 μm in diameter) located dorsally and ventrally. In the pedal ganglia, a big cell (70 μm) occurs on each side of the midline on the anterior ventral part and many cells of 40–50 μm form groups especially on the ventral surface.

FMRFamide immunoreactivity: Up to 250 immunoreactive perikarya were counted in the ganglia and many fibers were seen in the connectives and commissures (Figs. 3, 4). In the cerebral ganglia, cells of various sizes are abundant in the meta- and mesocerebrum. In each mesocerebrum, a big immunoreactive cell (80 μm) is located just posterior to the inner part of the cerebral lobe. In the CeGC area, many cells of medium size (40–50 μm) stain positively with the anti-FMRFamide serum. They send axons in different directions,



FIGS. 1–4. Maps of dorsal (Figs. 1, 3) and ventral (Figs. 2, 4) halves of central nervous systems of *Helix aspersa*. The perikarya (irregular circles) and fibers (dots and short lines) immunoreactive to anti-methionine-enkephalin (Figs. 1, 2) or anti-FMRFamide (Figs. 3, 4) are shown. C: cerebral ganglia; CeGC: cerebral green cells; DBa: dorsal bodies area; P: pedal ganglia; PA: parietal ganglia; PL: pleural ganglia, V: visceral ganglion. ×70.



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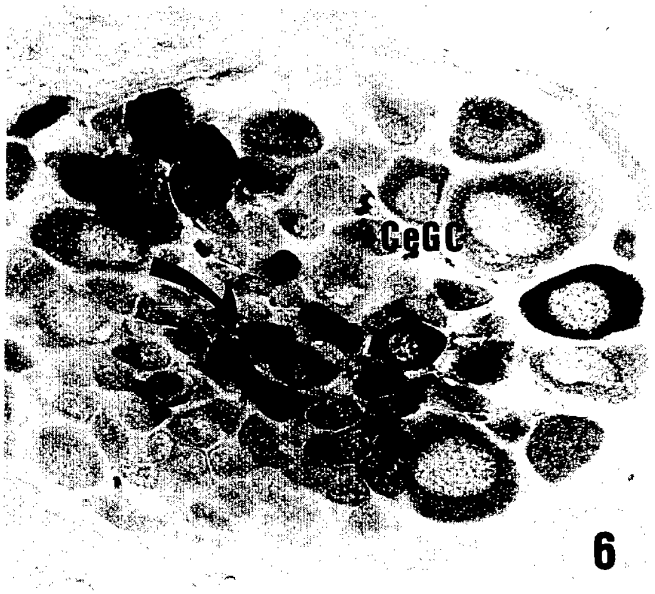

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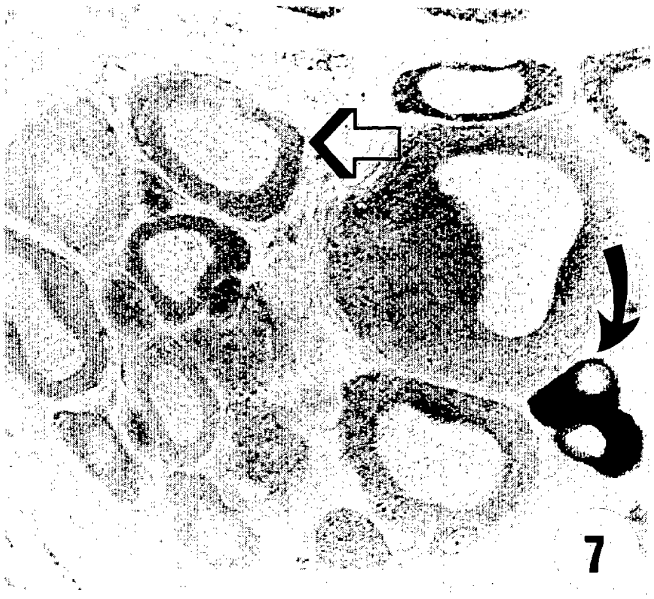

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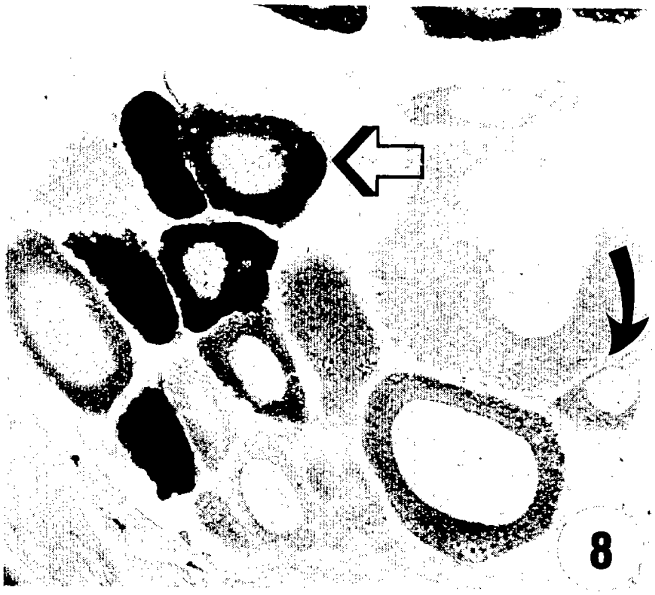

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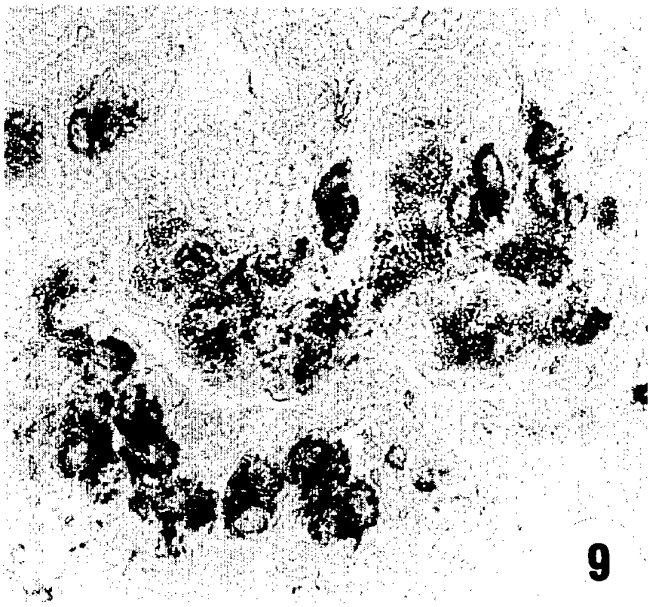

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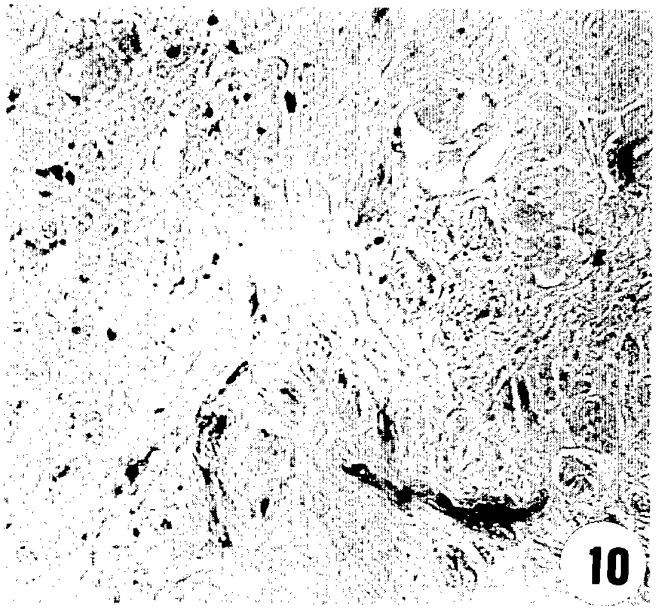

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but particularly through the intercerebral commissure (Fig. 12). The biggest perikarya (100–120 μm) are located ventrally in the visceral and parietal ganglia (Fig. 4). Whatever the conditions, there are little variations of the numbers of FMRFamide positive cells in all ganglia and especially in the two mesocerebral lobes which never contain FMRFamide positive perikarya. Adjacent sections treated either with anti-methionine-enkephalin or with anti-FMRFamide show that the two peptides are never colocalized (Figs. 5, 6, 7, 8, 9, 10).

Extra-ganglionic immunoreactive structures

Methionine-enkephalin immunoreactivity: The intra-connective endocrine cells (dorsal bodies) scattered in the vicinity of the anterior part of the supra- and subesophageal ganglia specifically bind the anti-methionine-enkephalin (Fig. 9). Sometimes, a large positive fiber is seen among them in the supra-esophageal area (Fig. 11). We have never observed methionine-enkephalin immunoreactive fibers in the subesophageal area.

FMRFamide immunoreactivity: Numerous small immunoreactive fibers are visible among, and often in contact with, the dorsal body cells in the supra- (Figs. 10, 13, 14) as well as in the subesophageal (Figs. 15, 16) perineurium of the central nervous system. They form a wide spread network in the dorsal body area. The use of immunogold technique confirms at the ultrastructural level the presence of FMRFamide-like substances in many nerves of the dorsal body area (Fig. 17) and in contact zones (synapse-like structures) between these nerves and the dorsal body cells (Fig. 18).

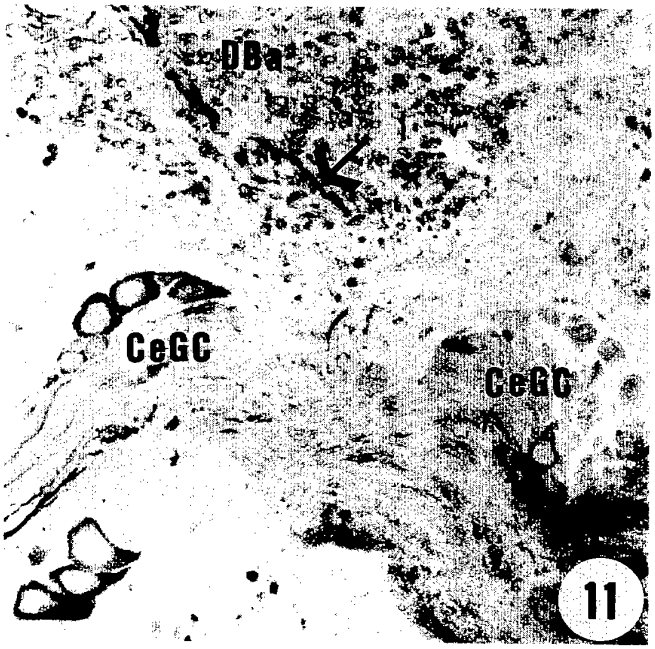
DISCUSSION

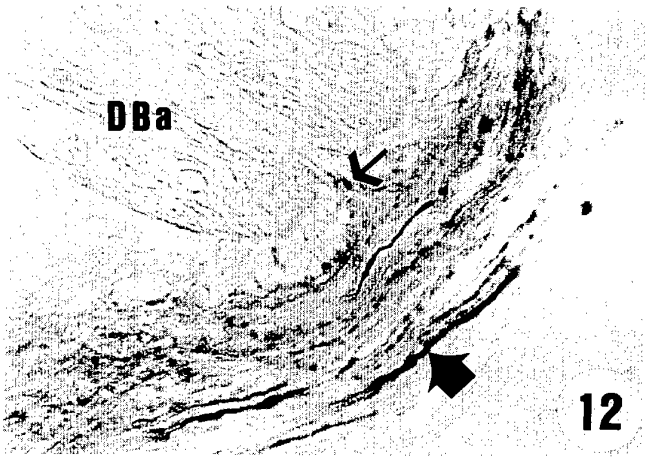
This study shows the important distribution of two substances immunologically detected with antibodies to, respectively, methionine-enkephalin and FMRFamide in perikarya and fibers of the central nervous system of *Helix aspersa*. It confirms previous observations made on different organs (aorta, gonad, nervous ring) [14, 15, 19, 21]. It also shows that methionine-enkephalin- and FMRFamide-like substances are never colocalized, either in the neurons, or in the dorsal bodies. At the present time, all the animals observed in any physiological situation (young, adult, before or after mating) never colocalize methionine-enkephalin and FMRFamide in their neurons.

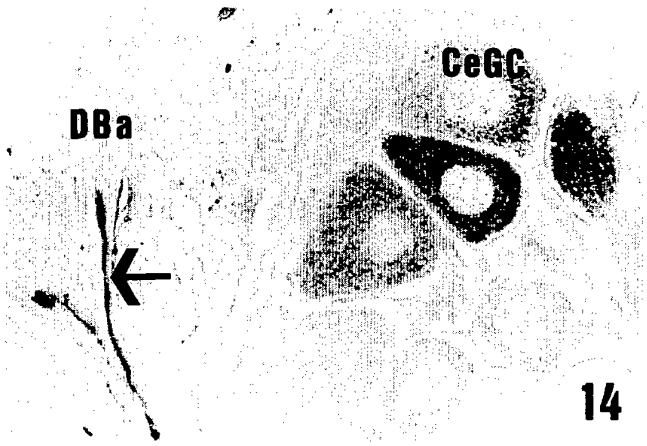
In comparison with histochemical data, immunocytochemistry gives additional information: e.g., some unstained cells in the pedal ganglia [3] are immunoreactive and are probably neurosecretory cells. Concerning the CeGC, the distribution of immunoreactive cells partially overlaps the distribution of alcian blue-alcian yellow positive cells. Immunocytochemistry however demonstrates the heterogeneity of the CeGC groups as some cells are methionine-enkephalin positive, other are FMRFamide immunoreactive and other are negative toward both antibodies; moreover the occurrence of insulin-related substances in these cells has been recently reported [22, 23]. This is worth pursuing in depth (for example by electron microscopy), so that an exact typology can be made.

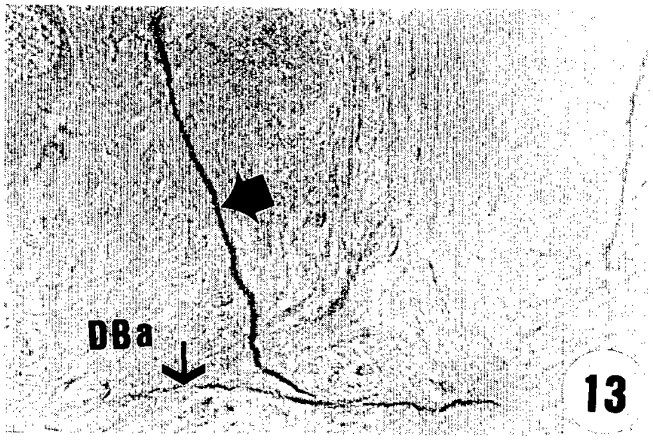
The large number of immunoreactive fibers and perikarya of various sizes in all ganglia probably reflects the diversity of functions of methionine-enkephalin- and FMRFamide-like substances in *Helix*. In molluscs, FMRFamide and related substances are known to play physiological roles in various tissues (cardiac and non cardiac muscles, nervous system, glands) and to act as neurotrans-

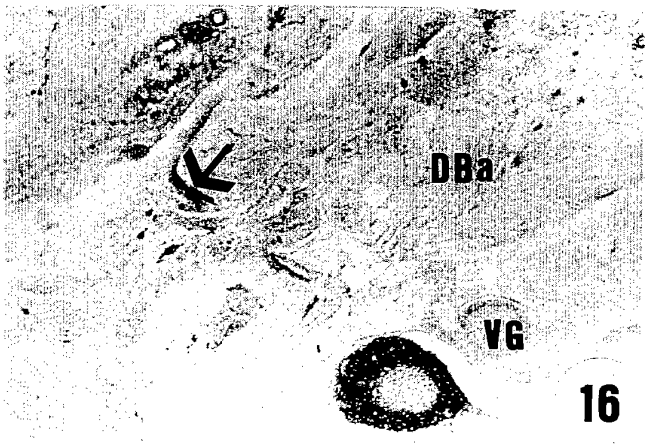
FIGS. 5–10. Immunocytochemical detection of methionine-enkephalin (Figs. 5, 7, 9) and FMRFamide-like material (Figs. 6, 8, 10) in the supra-esophageal (Figs. 5, 6), the subesophageal ganglia (Figs. 7, 8) and the dorsal bodies area located near the cerebral ganglia (Figs. 9, 10). (The arrows show the same area on adjacent sections). Figs. 5, 6. Adjacent sections to compare the localization of the immunoreactivity in the CeGC. ×290. Figs. 7, 8. Adjacent sections of the visceral ganglion. ×460. Neither the cerebral ganglia nor the visceral ganglion contain cells in which immunochemical staining for the two peptides-like is colocalized (Figs. 5, 6 and 7, 8). Fig. 9. The dorsal body cells are methionine-enkephalin immunoreactive. ×460. Fig. 10. The dorsal body cells do not react with the antibody raised against FMRFamide but they are innervated by FMRFamide positive fibers. ×480.

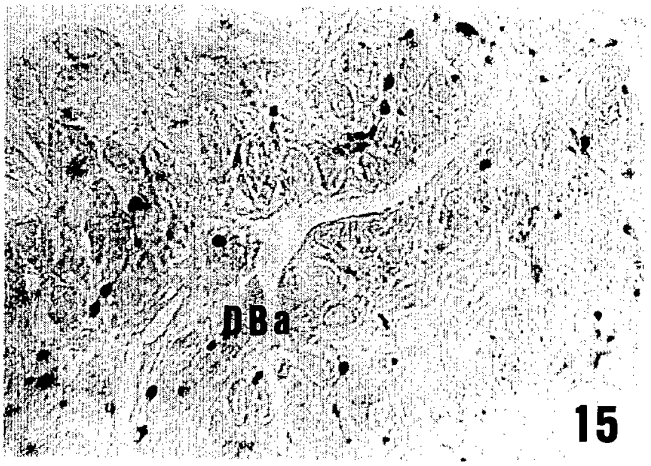


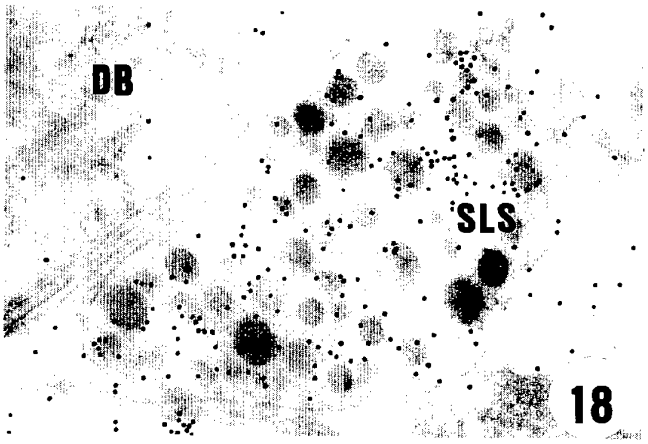


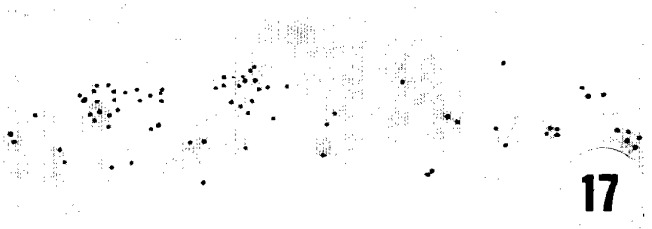












mitters-neuromodulators in the central nervous system, as neuro-muscular transmitters or as neurohormones [see 24–26]. Methionine-enkephalin, on the other hand, is involved in several processes such as locomotion, thermoregulation, pain resistance and immunomodulation [see 18].

Whereas the dorsal body cells of *Limax maximus* are immunoreactive to antisera exhibiting specificity for the C-terminal end of FMRFamide [27], those of *Helix aspersa* do not give positive reaction with our antiserum also raised against the C-terminal of FMRFamide. This divergent results might be due to the use of different polyclonal antisera or to specific differences in the secretion products of the dorsal body cells. The dorsal bodies of *Helix* give a very strong reaction with an antibody to methionine-enkephalin. Moreover, an important network of FMRFamide positive fibers is revealed, by immunocytochemistry, in the supra- as well as in the subesophageal dorsal body area. The supra-esophageal dorsal bodies seem to be controlled in part by FMRFamide-like substances, since synthetic FMRFamide and pQDPFLRFamide inhibit their *in vitro* protein synthesis [20, 28] and since FMRFamide-like substances are present in granules of many nerves and synapse-like structures of the dorsal body area [29]. The CeGC might exert this inhibitory control [10] and the present results are consistent with this notion because many CeGC are FMRFamide positive. However, our results further do not exclude the possibility that FMRFamide positive cells from other neuronal groups are also involved in the innervation of this area. In the same way it is logical to think that the majority of the FMRFamide positive fibers innervating the subesophageal dorsal bodies originate from the subesophageal ganglia as suggested by immunocytochemistry.

Finally, the nervous control of dorsal body cells is probably more complex than expected, particularly since we have revealed methionine-enkephalin positive fibers in the dorsal bodies in addition to FMRFamide positive ones; more recently Van Minnen and Schallig [22] have described MIP-(Molluscan Insulin Peptide) immunoreactive fibers in close association with the dorsal body cells of *Helix*.

In any case, it is difficult to follow the pathway of fibers from their origin to their end on serial sections treated by the immunocytochemical technics. Intraneuronal injections of lucifer yellow or other fluorescent substances will be helpful to precise where the dorsal bodies innervation comes from.

ACKNOWLEDGMENTS

The authors thank B. Jolibois for typing the manuscript and B. Régent for printing the photographs.

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FIG. 11. Immunocytochemical detection of methionine-enkephalin-like material in the supra-esophageal part of the nerve ring. The dorsal body area (DBa) is strongly methionine-enkephalin positive, as is a nervous fiber (arrow) traversing the area, probably coming from the cerebral ganglia, where some immunoreactive CeGC are visible. ×125.

FIGS. 12–16. Immunocytochemical detection of FMRFamide-like material in the supra-esophageal part of the nerve ring (Figs. 12, 13, 14) and near the visceral ganglion (Figs. 15, 16). Fig. 12 (×290) and 13 (×460). Path of FMRFamide positive fibers; among the intra-commissural positive fibers (large arrow), one is directed toward the DBa (small arrow). Fig. 14. Small CeGC of the right lobe and intra-DBa fibers (arrow) are FMRFamide positive. ×290. Fig. 15. The subesophageal DBa is innervated by FMRFamidergic axons (black points). ×460. Fig. 16. One might suppose that the FMRFamidergic fibers innervating the subesophageal DBa (arrow) originate from the visceral ganglia (VG). ×290.

FIG. 17. Anti-FMRFamide positive axon in the dorsal body area. Gold particles are concentrated on the granules of the axon. ×31000.

FIG. 18. Synapse-like structure (SLS) between a small nerve and a dorsal body cell (DB). The gold particles are very numerous on the granules of the SLS whereas the cytoplasm of the DB cell is not labelled. ×29300.

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