

## Distribution of $\alpha$ CDCP-immunoreactive neurons in the central nervous system of the snail *Helix aspersa*

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**Summary** —  $\alpha$ CDCP is a neuropeptide produced by the caudodorsal cells of *Lymnaea stagnalis* and encoded by the genes of the egg-laying hormone (ELH). The use of a polyclonal antiserum raised against  $\alpha$ CDCP resulted in the detection of about 800 immunoreactive neurons in the parietal ganglia and a small population (60 cells) in the cerebral ganglia of *Helix aspersa*. As the genes of ELH are well conserved among the gastropod species, these data designate the parietal ganglia as a putative source for the egg-laying hormone in *Helix aspersa*.

*Helix aspersa* /  $\alpha$ -caudodorsal peptide ( $\alpha$ CDCP) / ovulation hormone / immunocytochemistry

**Résumé** — Localisation de substances apparentées à l' $\alpha$ CDCP dans le système nerveux central de l'escargot *Helix aspersa*.  $\alpha$ CDCP est un neuropeptide synthétisé par les cellules caudodorsales de *Lymnaea stagnalis* et codé par les gènes de l'hormone de ponte (ELH). L'utilisation d'un antisérum anti- $\alpha$ CDCP démontre la présence d'environ 800 neurones immunoréactifs dans les ganglions pariétaux et d'une soixantaine de cellules positives dans les ganglions cérébroïdes d'*Helix aspersa*. Étant donné que les gènes de l'ELH sont bien conservés parmi les gastéropodes, ces résultats suggèrent que les ganglions pariétaux sont la principale source de l'hormone de ponte chez *Helix aspersa*.

*Helix aspersa* /  $\alpha$ CDCP / hormone d'ovulation / immunocytochimie

## INTRODUCTION

The neurosecretory caudodorsal cells (CDC) of the basommatophoran snail *Lymnaea stagnalis* produce a number of peptides among which the best studied is the ovulation hormone CDCH evidenced by Geraerts and Bohlken (1976). The ovulation hormone gene family is subdivided into 2 classes, the CDCH-I and CDCH-II genes (Vreugdenhil, 1988). Both genes are expressed in all CDC. They display over 90% homology and encode a different but overlapping set of neuropeptides which are supposed to be involved in various aspects of the control of ovulation and associated behaviour (Geraerts *et al*, 1988).  $\alpha$ CDCP is one of these peptides, derived from both CDC precursor proteins and consisting of 9 amino acid residues; it shows a high homology with the  $\alpha$ -bag cell peptide ( $\alpha$ BCP) region of the egg-laying hormone (ELH) precursor in the opisthobranch *Aplysia californica* (Vreugdenhil *et al*, 1988) and is thought to play an important role in the control of egg-laying (Van Minnen and Schallig, 1990a); moreover, it is involved in autoexcitation of the CDC (Brussaard *et al*, 1990). Preliminary investigations using an antiserum raised against  $\alpha$ CDCP showed the presence of positive cells in the visceral ganglion of *Helix aspersa* (Van Minnen and Schallig, 1990a). As very little is known about egg-laying control in the stylommatophora, and as previous results indicate a phylogenetically close relation between the ELH producing systems of gastropods (Van Minnen and Schallig, 1990a), it was considered that immunocytochemistry might be an interesting way to identify the neurons of *Helix aspersa* which are homologous to the CDC of *Lymnaea stagnalis* and the bag cells of *Aplysia californica*. The aim of this study was to provide a precise map of the  $\alpha$ CDCP immunoreactive structures and to

collect some indications on the putative ELH producing neurons in the central nervous system (CNS) of the garden snail. The results demonstrated that further experimental and molecular investigations on the ovulation hormone in *Helix aspersa* should focus on the parietal ganglia.

## MATERIAL AND METHODS

### Animals

Twelve adult specimens of the snail *Helix aspersa* were used. They were bred at the Centre Universitaire d'Héliciculture in Besançon, under controlled conditions (18 h light/6 h dark; 20 °C and 100% humidity). The CNS were dissected out and immediately transferred to the fixative for immunocytochemistry (see below).

### Production of the antiserum

The antibodies were raised in rabbits to the synthetic nonapeptide  $\alpha$ CDCP (EPRLRFHDV). They were prepared and tested for specificity at the Department of Biology of the Free University of Amsterdam, according to a previously described procedure (Van Minnen *et al*, 1988, 1989).

### Immunocytochemistry

The CNS were fixed for 24 h in Bouin-Hollande's 10% sublimate fixative. After dehydration, they were embedded in paraplast. Six- $\mu$ m serial sections were mounted on slides and processed for immunocytochemistry by the PAP method. They were incubated overnight at 4 °C in the primary antiserum (anti- $\alpha$ CDCP; dilution 1/1 000). The slides were washed twice in phosphate-buffered saline (PBS, pH 7.4), treated for 1 h at room temperature with the second antiserum (goat anti-rabbit, dilution 1/200) followed by peroxidase-antiperoxidase solution (dilution 1/100, duration 1 h, room temperature). The per-

oxidase was visualized with 0.05% 3,3' diaminobenzidine tetrahydrochloride in PBS containing 0.01%  $H_2O_2$ . Staining specificity was assessed by replacing the primary antiserum with preimmune serum or with antiserum preabsorbed with  $\alpha$ CDCP (2 mg for 0.5 ml of undiluted antiserum).

## RESULTS

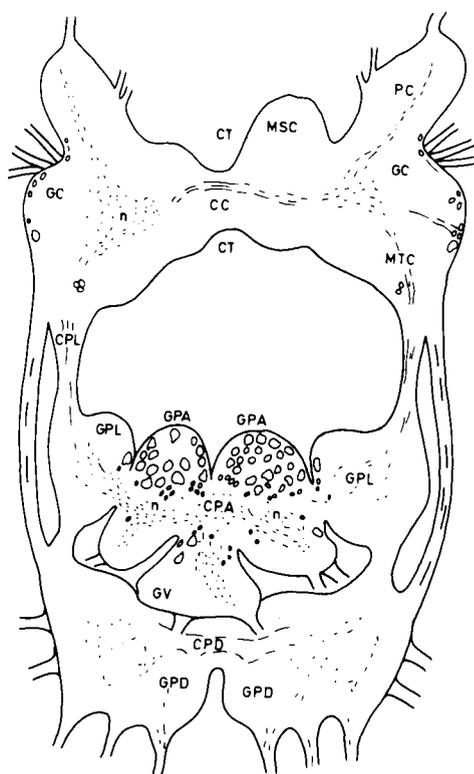
Our observations have been summarized in figure 1. Immunoreactive neurons were present in all ganglia except the pedal ganglia. Most of them were grouped in the suboesophageal nervous mass. The staining was distributed in the perikarya and axons and never in the nuclei. No immunoreactivity was observed following the control tests.

### Cerebral ganglia

The cerebral ganglia contained a few small immunoreactive neurons which were symmetrically distributed. A group of 3–5 weakly stained neurons ( $\varnothing = 15 \mu m$ ) was observed in the metacerebrum proximal to the origin of the cerebro-pleural connective (fig 2). Twenty-seven to 30 elongated strongly immunoreactive small neurons (widest diameter  $15 \mu m$ ) extended along the lateral external margin of the posterior procerebrum and the metacerebrum (fig 3). Their axons were directed towards the cerebral neuropil. In each ganglion, one larger positive cell ( $25 \mu m$  in diameter) was also found close to the external margin of the metacerebrum (fig 2).

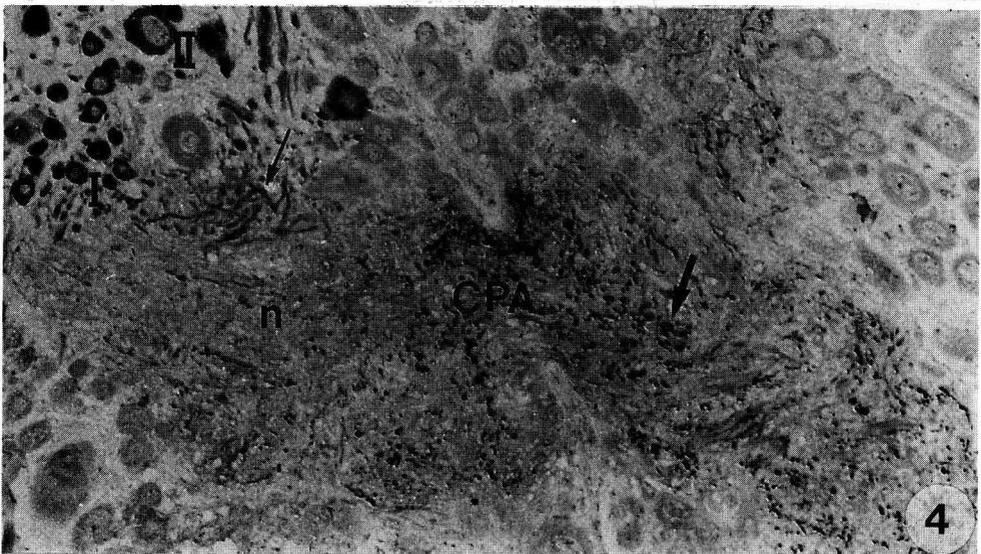
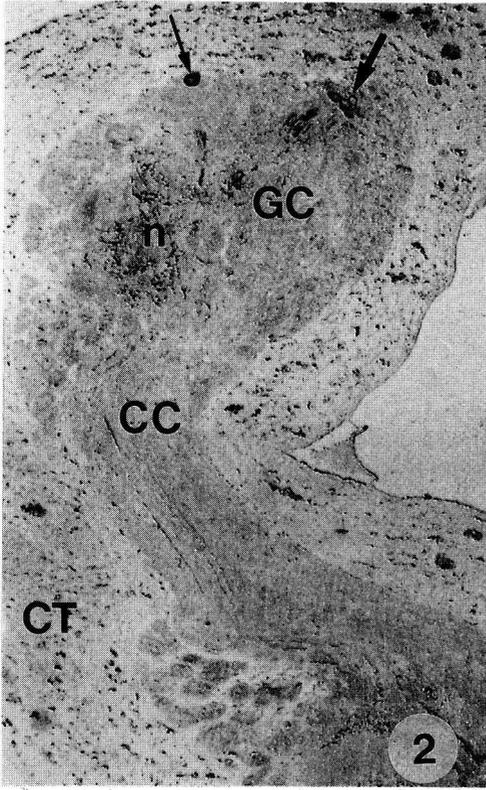
### Parietal ganglia

A large number of immunoreactive perikarya of various sizes were found in each parietal ganglion. Their distribution was not



**Fig 1.** Schematic horizontal representation of the localization of  $\alpha$ CDCP-immunoreactive cells (irregular circles) and fibres (short lines) in the *Helix* nervous system, based on reconstructions of serially-sectioned material. CC: cerebral commissure; CPA: parietal commissure; CPD: pedal commissure; CPL: cerebro-pleural connective tissue; CT: connective tissue; GC: cerebral ganglia; GPA: parietal ganglia; GPD: pedal ganglia; GPL: pleural ganglia; GV: visceral ganglion; MSC: mesocerebrum; MTC: metacerebrum; n: neuropils; PC: procerebrum.

symmetrical but they were more numerous in the right ganglion. Two different populations were distinguished. The more prominent (type I) consisted of about 480 cells measuring  $40\text{--}50 \mu m$  in diameter (290



cells in the right ganglion, 190 in the left). They were grouped in clusters and were surrounded or interspersed with non-immunoreactive cells. Their perikarya were moderately stained. The second population of immunoreactive neurons (type II) was made up of approximately 270 small (15–20  $\mu\text{m}$ ) strongly labelled cells (fig 6). These cells were preferentially located near the parietal commissure, especially in the anterior region (150 cells in the right ganglion and 120 in the left). They were either dispersed or loosely clustered. The giant neurons were never positive (fig 5).

### ***Pleural ganglia***

Only a few cells located at the margin close to the parietal ganglia were labelled (fig 7). Their features were similar to those of the immunoreactive cells in the parietal ganglia: 1 cell of type I and 4 to 6 neurons of type II were usually found in each ganglion.

### ***Visceral ganglion***

Labelled cells were scarce and situated in the anterior half of the ganglion. They con-

sisted of 4–5 type I cells and about 20 type II cells (fig 8). In addition, 18–20 small type II neurons were scattered between the fibres of the neuropil close to the parietal commissure.

### ***Pedal ganglia***

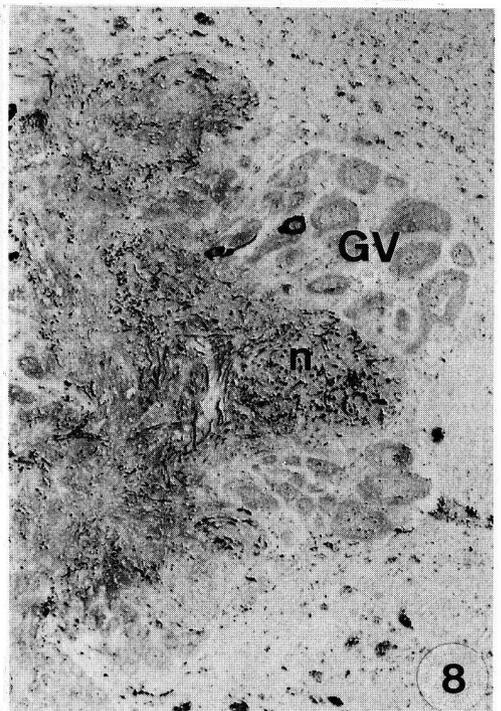
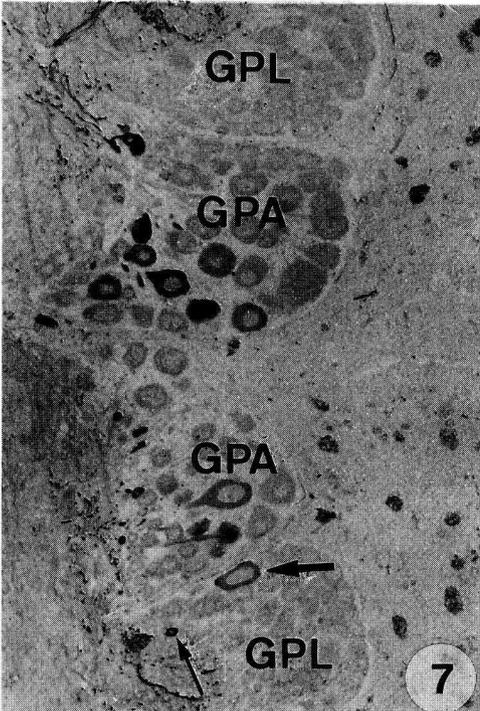
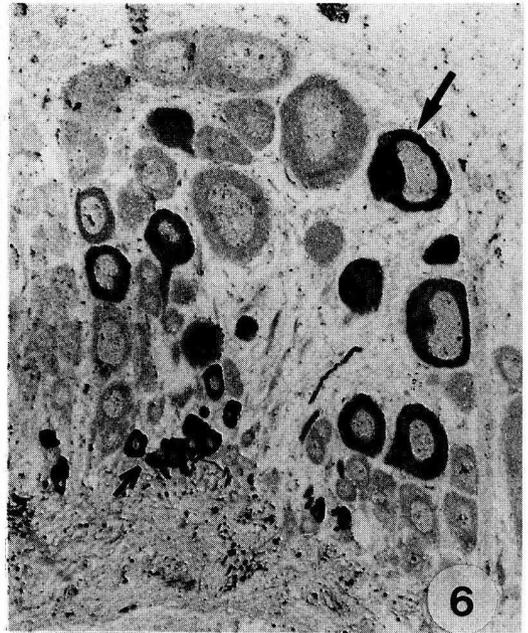
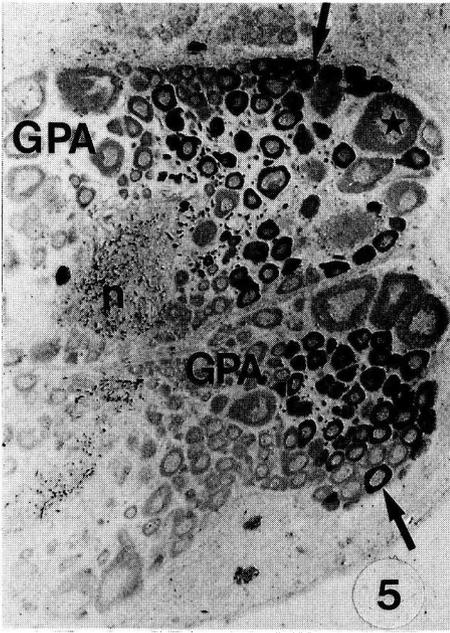
No positive perikarya were observed in these ganglia.

### ***Immunoreactive fibres***

An extensive network of immunoreactive fibres was visible throughout the CNS. Non varicose and varicose fibres were detected in the neuropil of all ganglia, including the pedal ganglia, in all commissures and connectives. They were particularly abundant in the parietal neuropils and commissure (fig 4). They were more discrete in the cerebral ganglia where a well defined bundle of fibres ran between the procerebral neuropil and the internal layer of globineurons (fig 3). Positive fibres were also detected in all peripheral nerve roots originating in the CNS.

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**Figs 2–4.** Examples of the distribution of immunoreactive elements in the supra- and suboesophageal ganglia. **2.** Positive cells in the left cerebral ganglia (GC): a small group of positive neurons (diameter = 15  $\mu\text{m}$ ) is found in the metacerebrum at the origin of the cerebro-pleural connective (large arrow). A larger cell (diameter = 25  $\mu\text{m}$ ) is visible in the lateral margin (thin arrow); CC: cerebral commissure; CT: connective tissue; n: neuropil; x 80. **3.** Left cerebral ganglion showing some small elongated immunoreactive cells (large arrow) located in the external margin of the procerebrum. Positive fibres are particularly visible lining the internal layer of globineurons (small arrow); x 85. CT: connective tissue. **4.** Neuropils (n) and parietal commissure (CPA). Immunoreactive non-varicose (thin arrow) or varicose (large arrow) fibres form a dense network. I: type I immunoreactive neurons; II: type II immunoreactive neurons in the right parietal ganglion; x 155.



## DISCUSSION

This study has confirmed the presence of  $\alpha$ CDCP-like substances in the CNS of *Helix aspersa*, and has determined their localization. Compared to the widespread distribution of other substances related to peptides of invertebrates or vertebrates, such as FMRFa or methionine-enkephalin (Marchand *et al*, 1982, 1991; Marchand and Dubois, 1986), the neurons reacting with anti- $\alpha$ CDCP are grouped. Most of them (about 800) were counted in the anterior part of the parietal ganglia or in the close vicinity. In the remainder of the CNS, about 30 neurons reacting with the antibody occur in each cerebral ganglion. This concentrated distribution is consistent with a well defined physiological role of the  $\alpha$ CDCP-like substances.

In *Lymnaea stagnalis*, distinct categories of cells contain CDCH-like peptides, the CDC and small intensely stained neurons in the lateral part of the cerebral ganglia, which are thought to be involved in the control of the CDC activity (Van Minnen *et al*, 1988). In *Helix* as in *Lymnaea*, 2 populations of neurons are specifically stained with the anti- $\alpha$ -CDCP; these 22 populations are distinguished by their staining and their size: one is characterized by large and moderately stained cells (described as type I in *Helix*), and the other by small and intensively stained cells (type II). However, the localization is not the

same in the 2 molluscs: the 2 kinds of neurons are located in the cerebral ganglia in *Lymnaea*, in the suboesophageal ganglia in *Helix* where they appear to be more numerous in the parietal ganglia.

The few immunoreactive neurons observed in the pro- and metacerebrum of *Helix* are morphologically more similar to type II, but not identical to it. They are not found in the mesocerebrum, and thus differ from the "cerebral green cells" described by Wijdenes *et al* (1980) and involved in the regulation of the dorsal bodies (Vincent *et al*, 1984; Wijdenes *et al*, 1987).

The dissymmetry in the distribution of immunoreactive cells in the parietal ganglia is not surprising. A similar observation was made by Chase (1986) for the cerebral ganglia. This author reports that in the mesocerebrum the right lobe is invariably larger than the left. He suggests that this dissymmetry is related to the sexual function, all reproductive organs being located on the right side of the snail's body.

Recent data show that many families of neuropeptides are well represented throughout the animal kingdom. During evolution, the peptides might have diverged structurally but they conserve similarities (Kerkhoven *et al*, 1990). This is true, for example, for the insulin-like peptides (Van Minnen and Schallig, 1990b) and for the ovulation hormone and some other peptides encoded by the ELH genes

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**Figs 5–8.** Examples of the distribution of immunoreactive elements in the supra- and suboesophageal ganglia (figs 5 and 7: the right ganglia are located in the upper part and the left ganglia in the lower part of the micrographs). **5.**  $\alpha$ CDCP immunoreactive neurons in the parietal ganglia (GPA). Type I cells are very numerous (arrows) in the anterior part of the ganglia. Positive fibres are visible in the neuropil (n);  $\times 70$ . Asterisk: giant neuron. **6.** Left parietal ganglion: 2 types of immunoreactive cells are evident. Type I consists of large moderately stained cells (large arrow) whereas type II is made up of small intensely stained cells (thin arrow);  $\times 150$ . **7.** Type I (large arrow) and type II (thin arrow) cells are present in the left pleural ganglion (GPL) close to the parietal ganglionic margin (GPA);  $\times 110$ . **8.** A few positive cells are found in the visceral ganglion (GV); n: neuropil;  $\times 85$ .

of molluscs (Nambu and Scheller, 1986). By using  $\alpha$ CDCP antibodies, Van Minnen and Schallig (1990a) were able to identify neurons in several species of Basommatophora and Stylommatophora which are homologous to the ELH producing cells in *Aplysia californica* and *Lymnaea stagnalis*.

In Stylommatophora, the nature and origin of ELH are not known. Rare observations concern the slugs in which, according to Takeda (1977), injections of brain homogenates stimulate egg-laying. In snails, the dorsal bodies (DB) are thought to be involved in the processes of ovulation as DB homogenates causes amoeboid movements in oocytes *in vitro* (Saleuddin *et al*, 1983) but injections of DB extracts in mature animals have failed to trigger egg-laying (Griffond and Mounzih, unpublished results). We can suppose that at least some of the  $\alpha$ CDCP immunoreactive cells described in the CNS of *Helix aspersa* represent the source of the ELH in this species. From the literature, we know the description (number, size, localization) of the bag cells of *Aplysia californica* and the CDC of *Lymnaea stagnalis* (Joosse, 1964; Boer, 1965; Coggeshall, 1967). The morphological criteria of number and cell size led us to suggest that type I neurons of *Helix* suboesophageal ganglia constitute a good candidate for producing ELH, but this must be verified by further investigations (descriptive and quantitative electron microscopy, *in situ* hybridization).

The present localization by immunocytochemistry of cells containing  $\alpha$ CDCP-like material is an essential step for the characterization of the neurons expressing the ovulation hormone genes in *Helix aspersa*. It will serve as a reference for subsequent experimental and molecular studies.

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