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Immunohistological evidence of dopamine cells in the cephalic nervous system of the silkworm *Bombyx mori*. Coexistence of dopamine and α endorphin-like substance in neurosecretory cells of the suboesophageal ganglion

Satoshi Takeda[1], **Jean Vieillemaringe**[2], **Michel Geffard**[3], **and Christian Rémy**[2]

[1] Sericultural Experiment Station, Yatabe, Ibarabi, Japan;
[2] Laboratoire de Neuroendocrinologie, Université de Bordeaux I, UA 1138 CNRS, Talence, France;
[3] Institut de Biochimie Cellulaire et Neurochimie du CNRS et U 259 INSERM, Bordeaux, France

Summary. Evidence of dopamine cells in the brain and the suboesophageal ganglion of the silkworm *Bombyx mori* was obtained immunohistologically in larvae and pupae. From six to eight and eight (two symmetrical groups of four) immunoreactive cells are present respectively in median and lateral protocerebral areas of the brain. In the suboesophageal ganglion, two cell clusters with dopamine immunoreactivity were observed. There was no clear difference in the nature of the immunohistochemical reaction and the number of cells between diapause- and non-diapause-egg producers, in both brains and suboesophageal ganglia. By examination of adjacent sections, it was possible to show that dopamine-immunoreactive cells in larval suboesophageal ganglia also contain an α endorphin-like substance.

Key words: *Bombyx mori* – Cephalic nervous system – Dopamine – α Endorphin – Immunohistology

Substances related to vertebrate neuropeptides or amines have been identified in the central nervous systems of many invertebrates by means of immunohistochemical techniques. Neurosecretory products immunologically related to most vertebrate neuropeptides have been detected in insects (Rémy 1982). In the same way, antibodies raised against specific amines helped to detect, in the central nervous systems of some insects, the presence of dopamine (Vieillemaringe et al. 1984) and serotonin (Bishop and O'Shea 1983; Klemm 1983; Klemm and Sundler 1983; Nässel and Klemm 1983; Nässel et al. 1983; Klemm et al. 1984; Nishiitsutsuji et al. 1984; Schürmann and Klemm 1984; Taghert and Goodman 1984; Tyrer et al. 1984).

In the silkworm *Bombyx mori*, immunohistological investigations had previously been carried out only with anti-neuropeptide antisera. Neurones containing insulin-like, gastrin-like and pancreatic polypeptide-like substances were thus detected in the brain (Yui et al. 1980) and an α endorphin-like substance was identified in the suboesophageal ganglion (Rémy et al. 1979). We first tried to detect the presence of dopaminergic cells in the brain and the suboesophageal ganglion with anti-dopamine antisera. We also tried to determine if an aminergic substance was likely to interfere with the neuroendocrine mechanisms occurring in the production of diapause or non-diapause eggs. Larvae and pupae of *Bombyx* belonging to two different breeds (one developing into diapause-egg producers and the other into non-diapause-egg producers) were used in our study.

Send offprint requests to: Dr. J. Vieillemaringe, Laboratoire de Neuroendocrinologie, Avenue des Facultés, 33405 Talence Cedex, France

Materials and methods

Dopamine was identified by means of two different types of antibodies. The immunogens used were obtained by linkage of dopamine (DA) to protein carriers (BSA with formaldehyde; HSA or poly-L-lysine or hemoglobin, with glutaraldehyde). The preparation of immunogens, the immunization technique used on rabbits and the control tests concerning antibody specificity and affinity have been described previously (for anti-DA formaldehyde antibodies: Geffard et al. 1982, 1984a; for anti-DA glutaraldehyde antibodies: Geffard et al. 1984a). In order to be able to locate the presence of dopamine in tissues in the most conclusive way, the substance used in coupling (formaldehyde or glutaraldehyde) was incorporated in the fixatives.

Animals. Two breeds of the silkworm *Bombyx mori*, N_4 (polyvoltine) and hybrid $N_{137} \times C_{137}$ were used. All their larval instars were reared with artificial diets. The former was incubated during embryonic stages at 15° C in the dark and the latter was exposed to light at 25° C, to obtain exclusively non-diapause- and diapause-egg producers, respectively.

Tissue preparation and immunohistochemical procedures. For each of the two breeds used, from 5 to 7 cephalic nervous systems complexes of both larvae and pupae were quickly dissected in ice-cold fixative. Some of them were fixed in Bouin-Hollande fixative without acetic acid (to which was added a saturated solution of mercuric chloride and 1% of sodium metabisulfite) whose formaldehyde content had been raised to 12%. Others were immersed in the same fixative containing 0,1% of glutaraldehyde. Fixation was performed for 36 h at 4° C. After washing overnight in veronal buffer (diluted to 1/5, pH 7.2) and elimination of the mercuric chloride by iodide 70° ethanol, the specimens were

Table 1. Immunolocalization of dopamine- and α endorphin-like substances in neurosecretory cells of the cephalic nervous system of the silkworm *Bombyx mori*

Antibodies	Larvae			Pupae		
	Median proto-cerebral cells	Lateral proto-cerebral cells	Sub-oesopha-geal cells	Median proto-cerebral cells	Lateral proto-cerebral cells	Sub-oesopha-geal cells
Anti-dopamine formaldehyde antibody 1/1000 (Dr. M. Geffard)	+	+	+	+	+	+
Anti-dopamine glutaraldehyde antibody 1/3000 (Dr. M. Geffard)	+	+	+	+	+	+
Anti-α endorphin antibody 1/100 (Dr. M.P. Dubois)	0	0	+	N.T	N.T	N.T

+: positive immunoreaction; 0: negative immunoreaction; N.T: non tested antibody

rapidly dehydrated, embedded in paraffin and cut into 7 µm transverse serial sections. These sections were quickly rehydrated to avoid oxydation of the catechol moiety and incubated overnight at 4° C with anti-dopamine (DA) or anti-α endorphin antisera diluted in veronal buffer 0.1 M, pH 7.2 + protein carrier:

a) anti-DA-formaldehyde antiserum diluted 1/1000 + BSA 1‰, for tissues immersed in a fixative containing formaldehyde but deprived of glutaraldehyde;

b) anti-DA-glutaraldehyde antiserum diluted 1/3000 + HSA-poly-lysine-hemoglobin 1‰, for cephalic nervous systems fixed in a solution containing glutaraldehyde;

c) anti-α endorphin antiserum (courtesy of Dr. M.P. Dubois) diluted 1/100 + 2‰ HSA (the preparation of this antiserum has been previously described; Rémy et al. 1979).

After washing, the sections were incubated with fluorescein isothiocyanate-conjugated sheep anti-rabbit Ig immunoglobulins diluted 1/50, for 1 h at room temperature. Finally, they were immersed in veronal buffer, counterstained with Evans blue 1/1000 for 3 min, mounted in buffered glycerin and observed under a fluorescence microscope.

The various phases of immunoreaction were carried out, as much as possible, in the dark.

Specificity tests. Specificity of the antisera was checked 1) by omission of the primary antiserum or incubation in nonimmune rabbit serum; 2) incubation in anti-DA antisera inactivated by the antigen (10^{-3} M dopamine solution) or by the immunogen (10^{-5} M DA-F-BSA, DA-G-HSA, DA-G-poly-lysine, DA-G-hemoglobin solutions; 3) incubation in α endorphin antiserum inactivated with α endorphin (500 µg of antigen/ml of undiluted antiserum).

Results

The results obtained with both anti-dopamine antisera proved to be the same (Table 1). In larval brains, dopamine cells were located in the medial group of neurosecretory cells (pars intercerebralis) and in the lateral neurosecretory cell area of the protocerebrum. From 6 to 8 immunoreactive globe-shaped cells (mean diameter 20 µm) were observed in the pars intercerebralis, in the vicinity of the large A_1 type neurosecretory cells (Fig. 1). In each of the two symmetrical lateral areas, immunoreactivity was found in 4 pearshaped neurons (20 × 20 µm) (Figs. 2, 3).

In larval suboesophageal ganglia, dopamine immunoreactive cells were detected at the level of two cell clusters. The first cluster, which is anterior and median, consists of 3 subspheric perikarya (20 µm) usually in line. The second one, containing 4 cells (25 µm) is located further back, in the ventral part of the front third of the ganglion. The number and location of these dopamine containing suboesophageal cells correspond perfectly to the cells crossreacting with an α endorphin antiserum, previously described in *Bombyx mori* (Rémy et al. 1979). These perikarya were identifiable on 3 or 4 adjacent sections owing to their large size; the dopamine antisera and α endorphin antiserum were used on alternate sections, and immunofluorescent reactions occurred in both cases (Figs. 4, 5 and Table 1). It was thus demonstrated that the α endorphin immunoreactive cells also contain dopamine. However, we were not able to show a similar colocalization in the brain, since the α endorphin antiserum did not give a positive reaction.

Control tests carried out in brains and suboesophageal ganglia were all conclusive (Figs. 6, 7). In particular dopamine and α endorphin materials were not visible in adjacent sections treated with antigen-inactivated antisera. In the larvae studied, we did not observe any relevant histological differences concerning the intensity of the reaction, the number and localization of immunoreactive cells in the two breeds used (N_4 and $N_{137} \times C_{137}$).

Next, dopamine immunoreactivity was examined in pupae of both diapause- and non-diapause-egg producers five and seven days after larval-pupal ecdysis, respectively. In spite of remarkable morphological changes in cephalic nervous systems, the number of cells and their immunoreactivity did not markedly differ from those of larvae. Furthermore, there was also no difference in the criteria mentioned above between diapause- and non-diapause-egg producers.

Discussion

Immunohistochemical detection of dopamine in the central nervous systems of insects by use of antibodies raised against this amine (instead of antisera against catecholamine synthesizing enzymes) was recently performed in locusts with anti-dopamine formaldehyde antibodies (Vieillemaringe et al. 1984). These antisera allowed us to visualize dopamine in the brain and the suboesophageal ganglion of *Bombyx mori*. We have also used anti-dopamine glutaral-

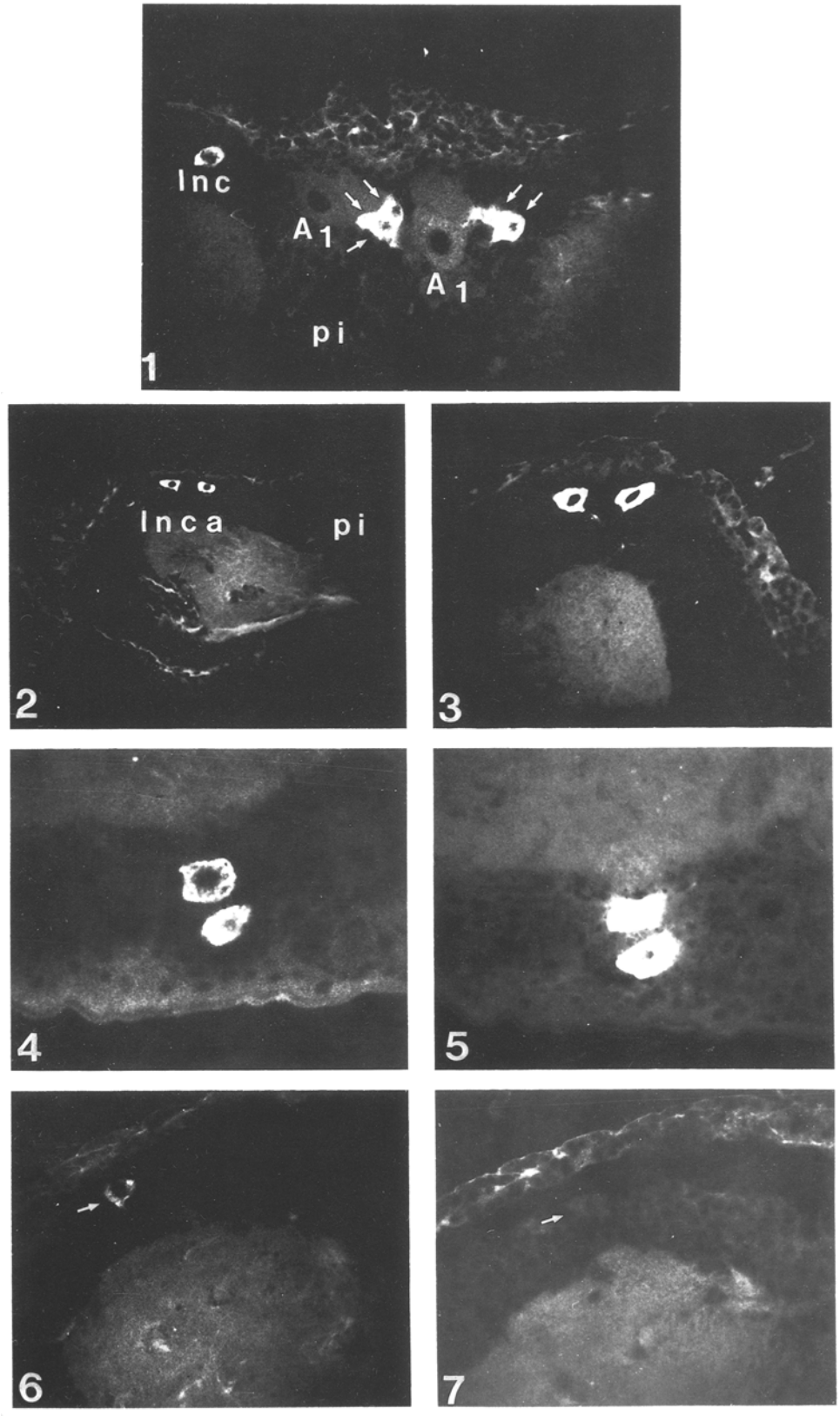

Fig. 1. Transverse section of larval protocerebrum treated with anti-dopamine antiserum. In pars intercerebralis (*pi*) five immunoreactive cells (*arrows*) near A_1 type neurosecretory cells (A_1). One reactive lateral neurosecretory cell (*Inc*) in left part of figure. × 250

Fig. 2. Larval protocerebrum. Two perikarya with dopamine-like content in lateral neurosecretory cell area (*Inca.*); *pi* pars intercerebralis. × 120

Fig. 3. Larval protocerebrum. Two dopamine immunoreactive cells in lateral neurosecretory cells area. × 250

Figs. 4–5. Two transverse adjacent sections of same larval suboesophageal perikarya (second cell cluster). **Fig. 4.** Section treated with anti-dopamine antiserum. × 350. **Fig. 5.** Section treated with anti-α endorphin antiserum. × 350

Figs. 6–7. Two adjacent sections of same lateral protocerebral perikaryon. **Fig. 6.** Section treated with anti-dopamine antiserum. One immunoreactive cell (*arrow*). × 250. **Fig. 7.** Adjacent section treated with anti-dopamine antiserum saturated with dopamine. No reaction at level of perikaryon (*arrow*). × 250

dehyde antibodies recently prepared and tested in vertebrates (Geffard et al. 1984a, b). They recognized dopamine with very high affinity and specificity at a low concentration (1/3000). The two classes of antisera localize dopamine in the same neurons of the cephalic nervous systems if the tissue is fixed by the homologous cross-linking agent.

There was essentially no difference in the nature of the immunohistochemical reaction and number of immunoreactive cells, in both brains and suboesophageal ganglia, between larvae and pupae and between non-diapause- and diapause-egg producers. Fukuda and Takeuchi (1967) insisted that the azocarmine positive cells located in the poste-

rior third of the suboesophageal ganglion, are identical with the diapause-factor producing cells. The suboesophageal dopamine reactive cells do not correspond with these azocarminophil cells, but with the α endorphin-like immunoreactive cells reported by Rémy et al. (1979). From the present results, we are not able to conclude if the dopamine containing cells play a role in the production of a neurohormone or of a neurotransmitter. It was suggested that the dopamine cells are not responsible for the important role of the regulation or the direct induction of egg diapause in the silkworm *Bombyx mori*.

The dopamine cells in the suboesophageal ganglion could be identified as the α endorphin immunoreactive cells already described (Rémy et al. 1979). In locusts, Vieillemaringe et al. (1982) demonstrated the coexistence of catecholamine and peptidergic neurosecretion in A type cells. The present results allowed us to show the colocalization of a peptide and an amine, for the first time, in lepidopteran neurosecretory cells. These observations are in agreement with recent results obtained in vertebrates and are inconsistent with the first part of Dale's principle "one neuron has the ability to synthesize only one transmitter".

In the pars intercerebralis of the silkworm, Kobayashi (1957) and Waku (1973) observed four pairs of large A_1 type neurosecretory cells (30×50 μm). Insulin-like immunoreactivity was found in these A_1 type cells (Yui et al. 1980). Dopamine immunoreactive cells were clearly distinguished from these A_1 type cells in both larval and pupal brains.

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