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Changes of Serotonin-Immunoreactive Neurons in Developing Larval Brains of Cabbage Butterfly *Artogeia rapae*

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This investigation was carried out to map the morphological development of serotonin-immunoreactive neurons in the larval brain of the cabbage butterfly, *Artogeia rapae*, during five larval stages. Both the first instar larva and the second instar larva contained twenty serotonin-immunoreactive (5-HTi) neurons in each brain. The fibres of 5-HTi commissure was interconnected to two cerebral hemispheres in both brains. However, the 5-HTi commissural fibres was increased in number in the second-instar larva brain. In the brain of the second instar larva these 5-HTi fibres formed rich arborization in contralateral neuropils, especially in the posterior parts of it. The third-instar larva brain, which included twenty two 5-HTi neurons, had three groups of 5-HTi commissural fibres. In the fourth instar larva, the number of 5-HTi fibres as well as 5-HTi cell bodies increased in the brain. The fifth-instar larva brain, which contained fifty four 5-HTi cell bodies, showed the largest number of 5-HTi cell bodies in developing larval brains. The 5-HTi fibres formed richest commissural fibres and some of them run parallel to anteroposterior axis.

KEY WORDS: Immunocytochemical Mapping, Serotonergic Neurons, Developing Larval Brains, Cabbage Worms

Evidence that serotonin (5-hydroxytryptamine, 5-HT) is distributed in the central nervous systems of the cockroach and other insects was given by quantitative fluorometric studies (Colhoun, 1963; Kusch, 1975; Evans, 1980). Additional evidences for the role of serotonin as a neurotransmitter were provided by its action on visceral and cardiac muscles of cockroach *Blaberas giganteus* and other insects (Cook *et al.*, 1969; Miller, 1979). Recently, immunocytochemical investigation on the central nervous systems of the insects revealed that brain contains serotonin as a neurotransmitter in locust *Schistocerca gregaria* (Tyrer *et al.*, 1984), in cockroach *Periplaneta americana* (Bishop and O'Shea, 1983), in honeybee *Apis mellifera* (Schürmann and Klemm, 1984), in blowfly *Calliphora erythrocephala* (Nässel and Laxmyr, 1983), and in cabbage butterfly *Artogeia rapae* (Lee *et al.*, 1992), and/or as a neurohormone in locust *Schistocerca gregaria* (Tyrer *et al.*, 1984) in migratory locust *Locusta migratoria* (Konings *et al.*, 1988), and in cabbage butterfly *Artogeia rapae* (Lee, 1994).

Many investigators have recently demonstrated that both larval and adult brains in the same insects produce serotonin as a neurotransmitter, using immunocytochemical studies (Nässel and Laxmyr, 1983; Lee *et al.*, 1992). Lee (1994) compared serotonin-immunoreactive (5-HTi) neurons in the pars intercerebralis between larval

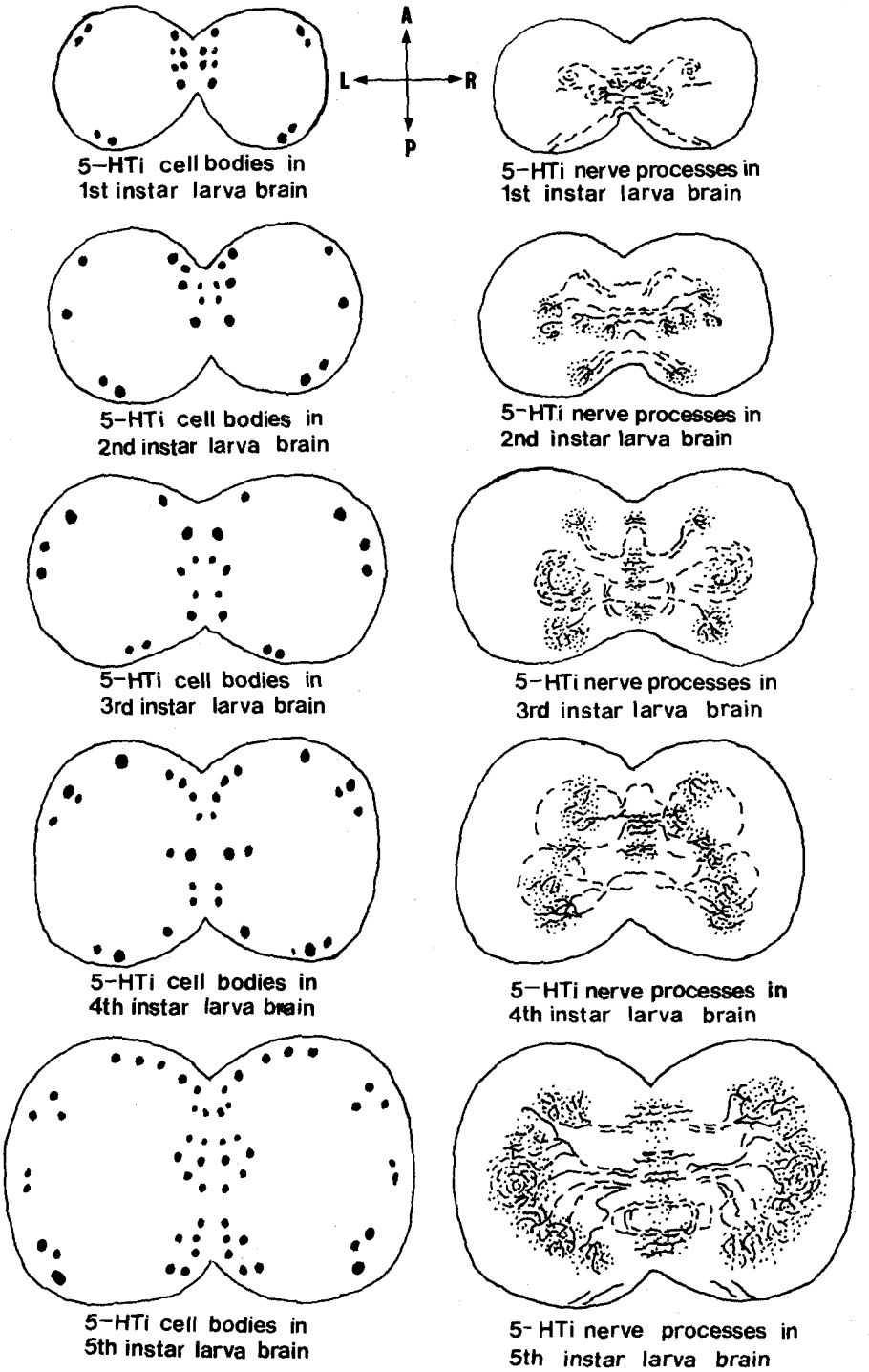

Fig. 1. Immunocytochemical mapping of 5-HTi neurons in developing brains of cabbage worm *Artogeia rapae*. The location of 5-HTi cell bodies and nerve processes in each developing brain shows bilateral symmetry. During development of the brains in first instar larva to fifth instar larva the 5-HTi neurons increase in number. A, anterior; P, posterior; L, left; R, right.

and adult brains of cabbage butterfly *Artogeia rapae*. However, it was very difficult to obtain experimental evidence on the production pattern of serotonin in developing larval brains of a insect.

The present paper describes mapping of serotonin-immunoreactive neurons in the developing larval brains of cabbage worm *Artogeia rapae*.

Materials and Methods

Adults of cabbage butterfly, *Artogeia rapae*, were collected from the fields near Seoul, and reared in outdoor rear facilities. They laid eggs on the leaves of Chinese cabbage. The eggs on the leaves developed to the larvae following certain given days. The first to fifth instar larva were used in this experiment.

The larvae were decapitated, and brains were immediately dissected in Ringer's solution and fixed in 4% paraformaldehyde in 0.01 M phosphate buffer, pH 7.2, for 1.5 h at 4°C.

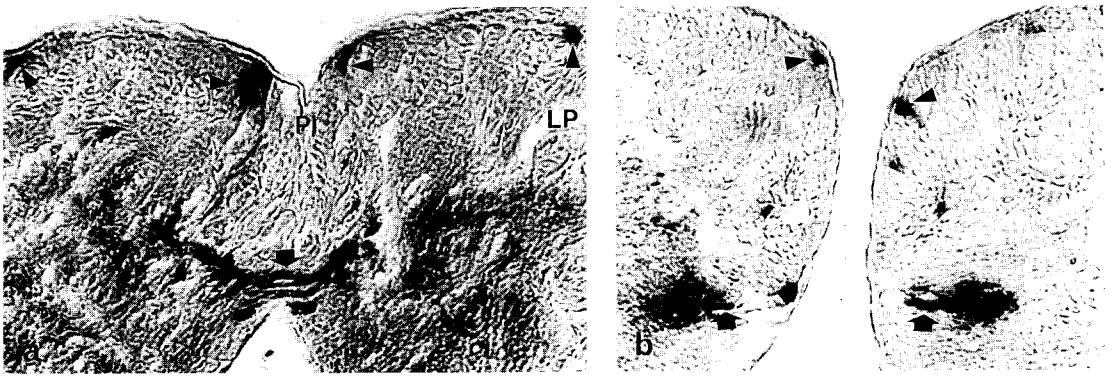

Fig. 2. Photographs of 5-HTi neurons in first-instar larva brain. (a) Note that 5-HTi cell bodies on the pars intercerebralis (PI) and lateral protocerebrum (LP) show bilateral symmetry in location. A 5-HTi commissural fibre bundle (arrow) interconnect between left and right hemispheres. × 250. (b) The 5-HTi cell bodies (arrowheads) in the pars intercerebralis and a part of a 5-HTi commissural fibre bundle (arrows) are seen. × 300.

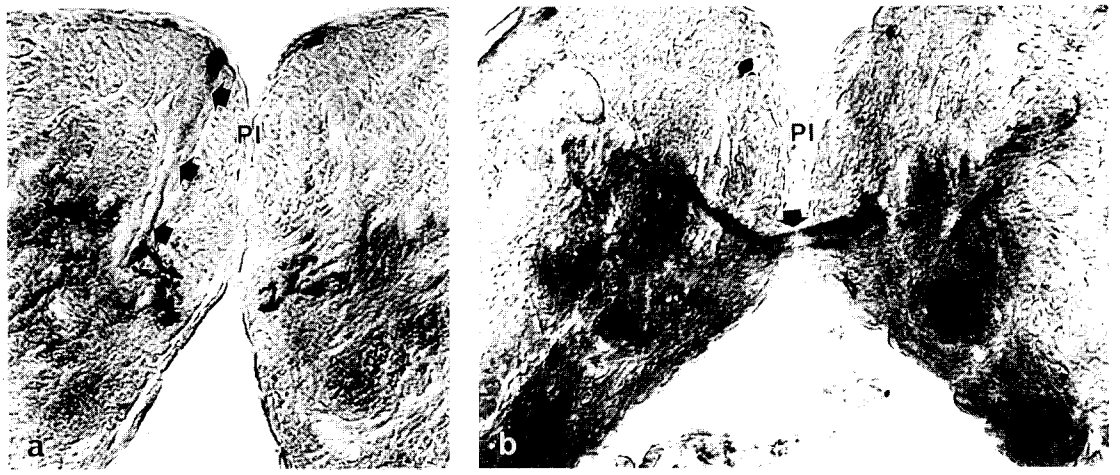

Fig. 3. Photographs of 5-HTi neurons in second-instar larva brain. (a) A pair of 5-HTi cell bodies are located in the pars intercerebralis (PI). One of them, located in left hemisphere, projects its axon (arrows) into medial, posterior area of the brain. × 300. (b) The 5-HTi commissural fibre bundles (arrow) are also found in second-instar larva brain. × 250.

Following washes in 0.01 M phosphate buffer for 1.5 h and dehydration in a series of graded alcohols, brains were embedded in paraffin. The serial sections cut at 7 μm were mounted on gelatin-coated slides.

The immunocytochemical staining of 5-HTi neurons in the brains was performed by avidin-biotin-peroxidase complex (ABC) method of Hsu *et al.* (1981). The antisera against an immunogen obtained by coupling serotonin to bovine serum albumin (BSA) with paraformaldehyde were supplied by Incstar Co.. For immunocytochemical staining the serial sections were rehydrated, washed for about 20 min in PBS, pH 7.2, and treated with normal goat serum (1:70 in PBS) for 20 min at 25°C. The sections were then incubated in serotonin antiserum (diluted to 1: 1000 in PBS) with 1% BSA for 1 h at 25°C, washed in PBS for 3 × 10 min, and reacted with anti-rabbit IgG in PBS. The sections were treated with avidin-biotin-peroxidase complex (Vectar stain Lab) for 40 min at 25°C, and then reacted with 0. 06% diaminobenzidine (DAB) containing 0.015% H_2O_2 at 25°C. Following two 10 min washes in PBS and another wash in distilled water, the sections were dehydrated and mounted with euparal.

Results

1st-instar larva brain

The twenty 5-HTi cell bodies are found in the first-instar larva brain (Fig. 1). The twelve of twenty 5-HTi cell bodies are located in the pars intercerebralis, while the two are distributed in lateral protocerebrum of each cerebral hemisphere. In each posterior protocerebrum two 5-HTi cell bodies can be also identified. The 5-HTi nerve fibre projected from a cell body in the pars intercerebralis runs to a posterior area (Fig. 2a). The nerve fibres which project into the contralateral, central neuropil though a cerebral commissure form arborization (Fig. 2b).

2nd-instar larva brain

The second-instar larva brain also contains twenty 5-HTi cell bodies (Fig. 1), as in first-instar larva brain. The location of 5-HTi cell bodies is also very similar to that of those in the first-instar larva brain (Fig. 1). However, the 5-HTi nerve fibres slightly increases in number in the second-instar larva brain (Fig. 3). The larger number of 5-HTi commissural nerve fibres distributed in the central neuropil of each cerebral hemisphere form more abundant arborization (Fig. 3b), especially in the posterior parts of both central neuropils.

3rd-instar larva brain

The 5-HTi cell bodies of the third-instar larva brain slightly increase in number in comparison with those in second-instar larva brain. The third-

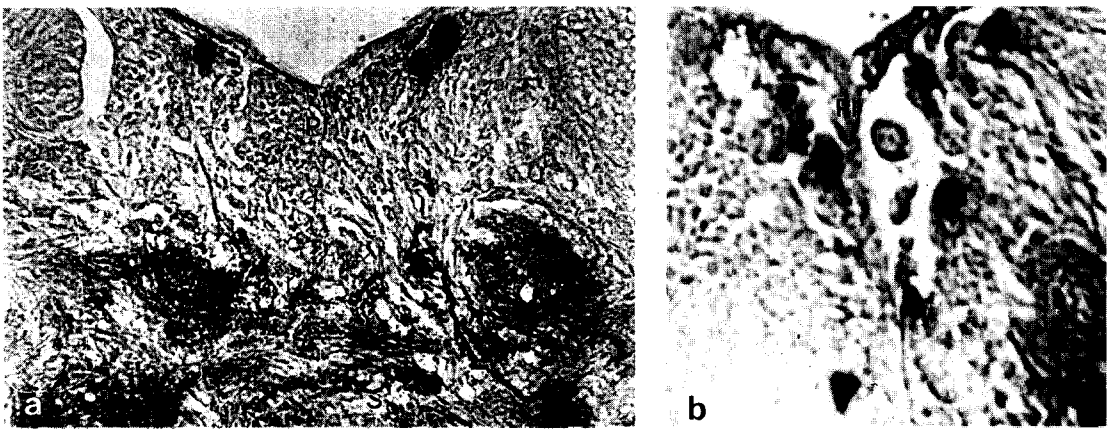

Fig. 4. Photographs of 5-HTi cell bodies in third-instar larva brain. The three (a) and eight (b) 5-HTi cell bodies are distributed in the pars intercerebralis (PI). (a) × 320. (b) × 400.

instar larva brain includes twenty two 5-HTi cell bodies (Fig. 1). Most of 5-HTi cell bodies in the third-instar larva brain are concentrated in the pars intercerebralis (Figs. 1, 4). The number of 5-HTi cell bodies which are located in lateral protocerebrum increase in the third-instar larva brain in comparison with those in the second-instar larva brain. The location of twelve 5-HTi cell bodies in the pars intercerebralis are also slightly changed in comparison with that in the second-instar larva brain.

4th-instar larva brain

The 5-HTi cell bodies of the brain initiate to increase greatly in number during the fourth-instar larval stage. In this developing stage the brain has thirty 5-HTi cell bodies. As shown in Fig. 1, the number of 5-HTi cell bodies increases in the pars intercerebralis and lateral protocerebrum, especially in the pars intercerebralis

As the 5-HTi cell bodies increases in number, the 5-HTi nerve fibres also show gradual increase in medial and central neuropils (Figs. 1, 5). In the central neuropil of each cerebral hemmisphere, all the 5-HTi nerve fibres form varicose arborization (Fig. 5a, c). The commissural fibres organized by 5-HTi nerve fibres continue to occur increasingly in medial region of the brain.

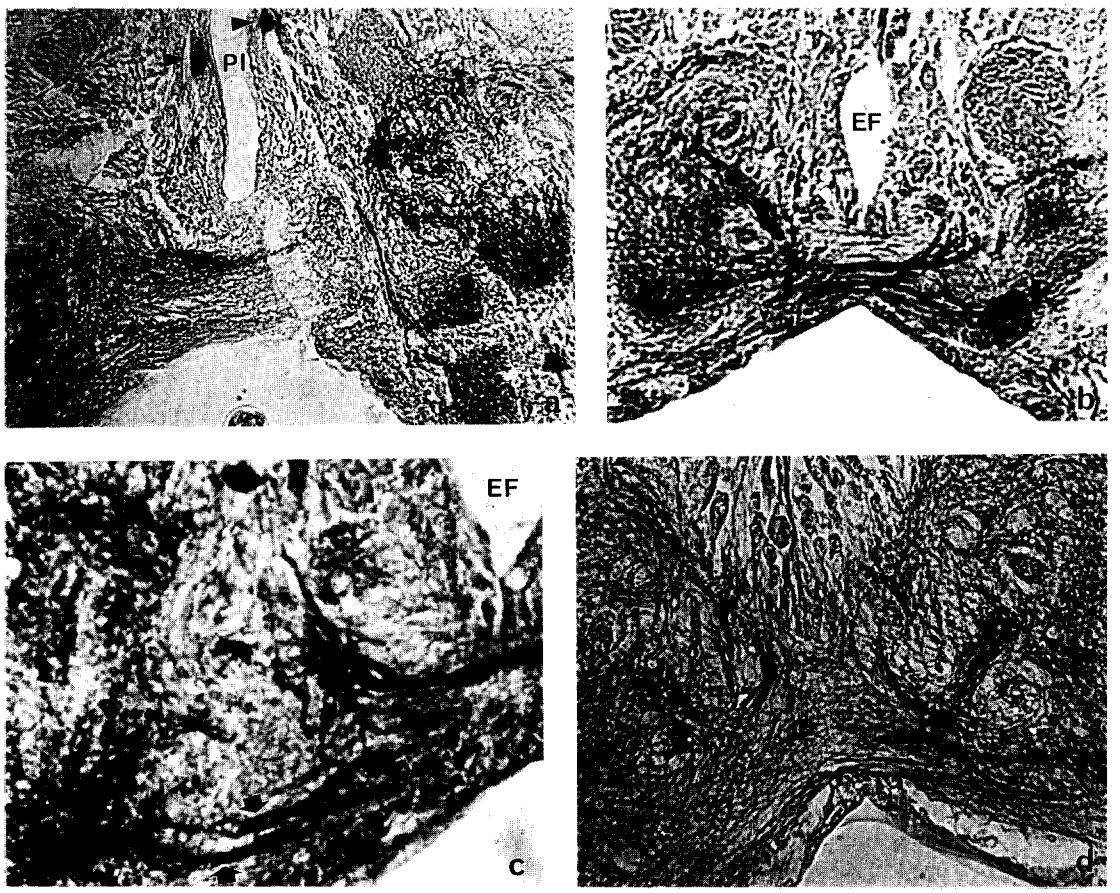

Fig. 5. Photographs of 5-HTi cell bodies and nerve processes in fourth-instar larva brain. (a) The brain contains two 5-HTi cell bodies (arrowheads) in the pars intercerebralis (PI) and abundant 5-HTi nerve processes. × 280. (b) The 5-HTi commissural nerve fibres are included in a cerebral medial region immediately posterior to esophageal foramen (EF). × 300. (c) The 5-HTi commissural nerve fibres (arrows) show varicose arborization in left cerebral hemisphere. EF, esophageal foramen. × 330. (d) The abundant 5-HTi nerve fibres run through the cerebral medial region. × 300.

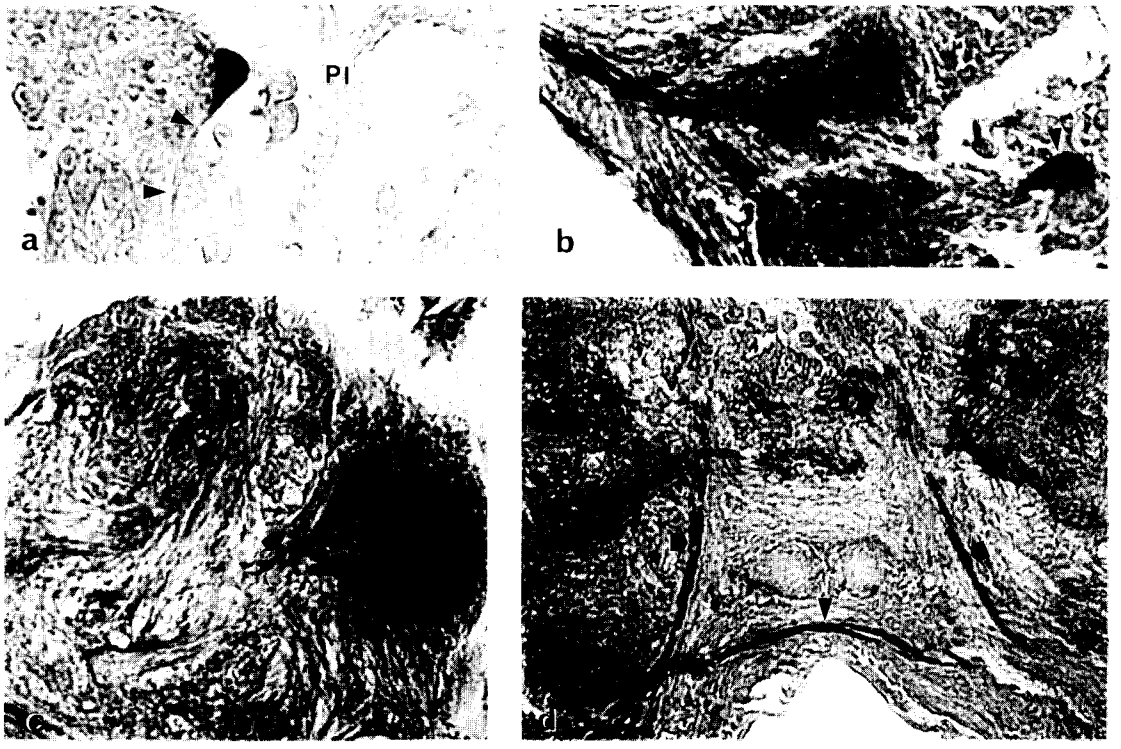

Fig. 6. Photographs of 5-HTi neurons in fifth-instar larva brain. (a) A 5-HTi neuron is found in the pars intercerebralis (PI). The axon (arrowhead) runs to the posterior direction of the brain. × 600. (b) A 5-HTi cell body (arrowhead) and the large 5-HTi commissural nerve fibre (arrow) are located in a posterior region of right cerebral hemisphere. × 600. (c) A 5-HTi nerve fibre shows varicose arborization (arrow). × 620. (d) The brain includes a large 5-HTi commissural nerve fibre (arrowhead) and two parallelly-running 5-HTi nerve fibres (arrows) in medial region. × 280.

5th-instar larva brain

The fifth-instar larva brain has greatly increased number of 5-HTi neurons. Especially in the pars intercerebralis, a large number of 5-HTi cell bodies are located: more than thirty 5-HTi cell bodies. The fifty four 5-HTi cell bodies are included in the pars intercerebralis and lateral protocerebrum of fifth-instar larva brain (Fig. 1). The 5-HTi cell bodies and nerve processes show strong immunoreactivities to anti-serotonin (Fig. 6).

The neural network formed by 5-HTi nerve processes in the neuropil of fifth-instar larva brain is the most complex in the developing larval brains. As shown in Fig. 1 and 6, the 5-HTi nerve processes constitute large volume of the cerebral neuropil (Fig. 6c, d). Many 5-HTi nerve fibres also form commissural fibres which interconnect between left and right cerebral hemispheres. Although most of the 5-HTi nerve fibres are commissural fibres which run parallel to left-to right axis, some of the 5-HTi nerve fibres also run parallel to anteroposterior axis (Fig. 6d).

Discussion

The developing larval brains in cabbage butterfly *Artogeia rapae* contain each about twenty 5-HTi neurons in first and second instar larva, about twenty two in third instar larva, about thirty in fourth instar larva, and fifty four in fifth instar larva. Lee *et al.* (1992) have shown that in the same insect the postembryonic brains have about fifty four 5-HTi neurons in fifth instar larva, about twenty in two-day-old pupa, and about one hundred eighteen in one-day-old adult. These experimental data imply that in postembryonic

development of cabbage butterfly, the number of the cerebral 5-HTi neurons gradually increase during larval life, and following decrease of them during pupal life, reincreases remarkably in early adult stage.

The 5-HTi neurons located in the brain are supposed to show their 5-HTi phenotype due to serotonin biosynthesis within the neuron during larval metamorphosis from the first instar larva to fifth instar larva. The 5-HTi neurons in insect brain were described to differentiate initially during embryonic development (Taghert and Goodman, 1984). It has also been mentioned that the 5-HTi neurons, which survive in larval brains after embryonic development, remain immunoreactive (Nässel, 1988). According to these experimental evidences, increase of 5-HTi neurons in the brain during larval metamorphosis can be due to development of more 5-HTi neurons in comparison with their degeneration. This larval 5-HTi system forms adult 5-HTi system following modification in the process of metamorphosis in pupa. (Lee *et al.*, 1992)

The morphological changes of many 5-HTi neurons resemble those of metamorphosing motorneurons in moths (Truman and Reiss. 1976). It is reasonable to assume the 5-HTi neurons of the fifth instar larva brain interact with neurons in circuit different from those of the first instar larva brain (Nässel, 1988). Hence, the change in morphology of the 5-HTi neurons may be accompanied by a change of thier funtional roles (Levine and Truman, 1981, Nässel, 1988).

The larval brain of the cabbage butterfly *Artogeia rapae* contains three cerebral 5-HTi commissures, which interconnect two cerebral hemispheres in third instar larva, in fourth instar larva, and in fifth instar larva. These 5-HTi commissures differentiate initially in the brain of the first instar larva. With the increase of developing days during larval life, the 5-HTi nerve fibres interconnecting the cerebral hemispheres increase in number. The larval brain of the insects, such as fly *Sarcophaga bullata*, also contains at least three 5-HTi brain commissures (Nässel *et al.*, 1986). According to immunocytochemical investigation of Lee *et al.* (1992) in *Pieris rapae*, three 5-HTi cerebral commissures were described to reduce in number to the two.

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발생중인 배추흰나비의 유충 뇌에서 세로토닌 면역반응성 신경원의 변화

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배추흰나비 유충 뇌에 분포하는 세로토닌 면역반응성 신경원(이하 세로토닌 세포)이 발생에 따라 형태학적으로 어떻게 분화해 나가는지를 조사하였다. 1령 유충뇌와 2령 유충뇌는 각각 20개의 세로토닌 세포를 포함하였다. 1령 유충뇌에서는 세로토닌 면역 반응성 섬유(이하 세로토닌 섬유) 한무리가 뇌교련을 형성하였고 이같은 섬유의 대부분은 반대쪽 중앙 신경망에 종지하였다. 2령 유충의 뇌에서는 세로토닌 섬유의 대부분이 뇌교련을 형성하였고 1령 유충뇌에서 보다는 그들의 수가 더 많이 관찰되었다. 이 섬유의 종말이 형성하는 보다 풍부한 arborization은 중앙 신경망의 상당한 부분을 차지하였다. 3령 유충뇌의 세로토닌 세포는 22개 였고 세로토닌 섬유들이 구성하는 뇌교련수도 3개로 증가되었으며 세로토닌 섬유의 대부분은 뇌교련을 형성하였다. 30개의 세로토닌 세포를 가진 4령 유충뇌와 54개의 세로토닌 세포를 가진 5령 유충뇌는 3개의 뇌교련을 구성하는 세로토닌 섬유뿐만 아니라 뇌의 전후 방향으로 달리는 세로토닌 섬유도 포함하였다.