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Postembryonic Development of Leucokinin I-Producing Neurons in the Brain of Insect *Spodoptera litura*

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Antisera against the myotropic neuropeptide leucokinin I, originally isolated from head extracts of the cockroach *Leucophaea maderae*, have been used to investigate the distribution of the leucokinin I-immunoreactive (LK I-IR) neurons in the brain of the common cutworm, *Spodoptera litura*, during postembryonic development. The LK I-IR neurons are found at the larval stages (excluding first instar larval stage), pupal stages, and adult stage, of which the brains have been examined in this experiment. The number of the LK I-IR neurons in the brain increases from the second instar larva to the fifth instar larva which has about 32, the largest number in all postembryonic stages. Thereafter, the LK I-IR neurons begin to decrease in number. During the pupal stages, smaller number of LK I-IR neurons persist in the brains; 6 or 4. At adult stage the brain contains 8 LK I-IR neurons. The LK I-IR cell bodies are distributed in each dorsal cortex of both cerebral hemispheres in the second instar larva and through all the neuromeres of the brain during later larval stages, despite of being a large number of the LK I-IR cell bodies in dorsolateral neuromeres. At pupal stages, most of the LK I-IR cell bodies are found in the pars intercerebralis. Extremely small number of the LK I-IR cell bodies are localized in the pars lateralis. Adult brain contains the LK I-IR cell bodies in the pars intercerebralis and the middle cortex of the posterior brain. The LK I-IR nerve processes can be easily found in the neuropils of almost all the neuromeres in the brains of third, fourth, fifth and sixth instar larvae. Most of the LK I-IR nerve fibers in those brains are originated from the LK I-IR cell bodies located in the brains. The LK I-IR cell bodies which have very weak reactivities to the antisera do not show projection of the LK I-IR nerve processes in the brains.

The leucokinins are insect neuropeptides that had been originally isolated in head extracts of the cockroach *Leucophaea maderae* (Holman et al., 1986a, b) and then in cricket *Acheta domesticus* and locust *Locusta migratoria* (Holman et al., 1990a, b; Schoofs et al., 1992). The octapeptides leucokinin I-VIII (LK I-VIII) comprise the largest family of insect kinins, distinct from the tachykinins (Nässel, 1993a; Meola et al., 1994). The LKs have myotropic action on insect visceral muscle. In addition to their myotropic action in insects, LKs have been found to affect ion transport in the Malpighian tubules (Holman et al., 1990a, b). In particular, LK VIII inhibited transepithelial secretion from the Malpighian tubules of mosquito *Aedes aegypti* at low concentration (10 pM), whereas it stimulated the secretion at high concentration (3.5 mM) (Hayes et al., 1989).

Immunocytochemical investigations on the LK I-immunoreactive (LK I-IR) neurons using antisera raised against LK I (Asp-Pro-Ala-Phe-Asn-Ser-Trp-Gly-NH_2) included their localization in the brain of the cockroach *Leucophaea maderae* (Nässel et al., 1992). Nässel et al. (1992) have demonstrated that the adult brain of *Leucophaea maderae* has about 160 LK I-IR neuronal cell bodies in the protocerebrum and optic lobes. Of about 160 LK I-IR neurons, both LK I-IR interneurons and LK I-IR neurosecretory cells, of course, are included. In the brain of the blowfly *Calliphora vomitoria* about 60 LK I-IR neurons have been shown to be localized in protocerebrum, deutocerebrum, tritocerebrum, optic lobe and suboesophageal ganglion (Nässel, 1993a).

In the cockroach *Leucophaea maderae,* large amount of leucokinin immunoreactivity was found in the corpora cardiaca-corpora allata complex, suggesting that LK could play a key role as a hormone (Muren et al., 1993). It has been also demonstrated that no differences in levels of the LK could be detected in any tissues, in comparison with male and female cockroaches (Nässel, 1993a). Chen et al. (1994a) have shown the localization of LK IV-IR neurons in central nervous systems of the cockroach *Nauphoeta cinerea*, cricket *Acheta domesticus*, mos-

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quito *Aedes aegypti*, locust *Schistocera americana* and honey bee *Apis mellofera*.

It has been shown that an antiserum raised against an achetakinin analog selectively localized leucokinin VIII in central nervous system of the cockroach *Leucophaea maderae* (Meola et al., 1994). Concerned with postembryonic development of LK-immunoreactive cells in nervous systems of the insects, two studies were done in the tobacco hornworm *Manduca sexta* (Chen et al., 1994b) and the turnip moth *Agrotis segetum* (Cantera et al., 1992). However, Chen et al. (1994b) concentrated on identification of the LK IV-IR neurosecretory cells in *Manduca sexta* that might be a source of leucokinin-like neurohormone. Cantera et al. (1992) focused mainly on postembryonic development of LK I-IR neurons innervating to neurohaemal organ in *Agrotis segetum*. Therefore, further studies on the changing pattern of LK I-IR neurons in insect brains at various stages of postembryonic development, in fact, are certainly necessary. In this paper, differentiation pattern of the LK I-IR neurons in changing brains at various stages of postembryonic development is described in the common cutworm *Spodoptera litura*, using specific antiserum against leucokinin I.

Materials and Methods

Experimental insects

First instar larvae of the common cutworm *Spodoptera litura* were obtained from stock colonies in insect growth chamber in Department of Biology, Korea University. The larvae were reared in a growth chamber controlled at about 25℃ under constant photoperiod(16 h light and 8 h dark cycle) at about 70% relative humidity and fed artificial diet. In growth chamber, the larvae were metamorphosed to the pupae which then become adults. The first instar, second instar, third instar, fourth instar, fifth instar and sixth instar larvae, prepupae, one-day-old, three-day-old, five-day-old and seven-day-old pupae, and one-day-old adults were collected from colonies in growth chamber, and used as experimental insects in this investigation.

Antiserum

The anti-LK I antiserum used as a primary antibody in this experiment was a generous gift from Dr. Nässel of Stockholm University. The detailed production procedure of anti-LK I was described by Nässel et al. (1992).

Immunocytochemistry

The head cuticle of adult of the common cutworm *Spodoptera litura* was opened by dissection in 0.1 M phosphate buffer (pH 7.4) at 4℃ and the whole brains were completely isolated from the heads. The brains were then fixed by immediate immersion into 4% paraformaldehyde in phosphate buffer for 12 to 16 h. The fixed brains were thoroughly washed several times in phosphate buffer. The brain tissues used for cryo-sectioning were immersed in 20% sucrose in phosphate buffer for 24 h. After being embedded in tissue-tek (Elkhart, IN) and frozen the stage of cryostat machine, the brains were sectioned at -14℃ and at 25 μm in thickness. The cryocut brain sections were put on slide glasses coated with chromium potassium sulfate and gelatin.

The immunostaining procedures were carried out according to descriptions by Nässel and Lundquist (1991). The brain sections on the slide glasses were briefly washed in phosphate buffer and wash buffer (0.1M phosphate buffered saline containing 0.25% Triton X-100). Thereafter, the brain sections were incubated with the primary antibody anti-LK I diluted to 1:100, 1:500, 1:1000, 1:2000 and 1:4000 in 0.1 M phosphate buffered saline with 0.25% Triton X-100 and 0.5% BSA for 48 h. at 4℃. For proper immunostaining, the dilution of the anti-LK I antiserum was shown to be 1:1000. After being thoroughly washed in wash buffer, the brain sections were reacted with the swine anti-rabbit IgG diluted to 1:50 for 2 h. at room temperature. The washing of the brain sections in wash buffer was subsequently performed, followed by reaction with 1:100 diluted peroxidase-antiperoxidase (PAP) of rabbit for 2 h. at room temperature. The washes in wash buffer and 0.05 M Tris buffer were continued, before stained with 0.03% diaminobenzidine (DAB) in Tris buffer containing 0.01% hydrogen peroxide. Following dehydration of brain sections with graded ethanol solutions (70% to 100%) and xylene, the brain sections were embedded in Permount. The results of immunostaining on the brain sections that had been mounted were examined by a light microscope and then photographed. Thereafter, the schematic drawings for localization of the LK I-IR cell bodies and their nerve fibers were made on the basis of the LK I-IR neural networks using camera lucida.

For the whole brain immunostaining, the following method of Nässel et al. (1992) was employed. The head cuticles of the common cutworm *Spodoptera litura* at various postembryonic developmental stages (first to sixth instar larvae, prepupae, one-, three-, five- and seven-day-old pupae, and one-day-old adults) were opened by careful removal of cuticle in 0.1 M phosphate buffer (pH 7.4) at 4℃ and the fixation, of the brains was initiated by immediate immersion into 4% paraformaldehyde in phosphate buffer at 4℃. Optimal fixation time was for 6 to 10 h. After the fixation, the insect whole brains were isolated by complete removing of the cuticle around the heads under a stereoscope. Thereafter experimental

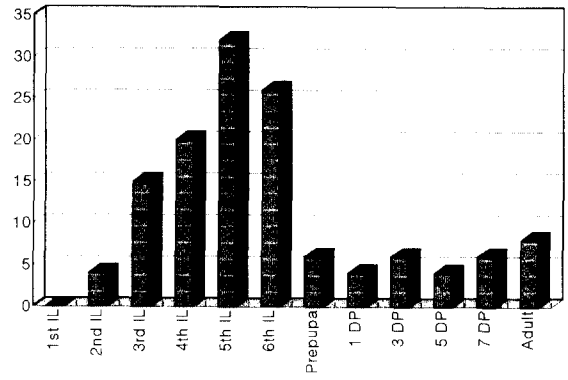

Fig. 1. Numerical changes of leucokinin I -immunoreactive neuron in brains at various postembryonic developmental stages of the common cutworm, *Spodoptera litura*. The LK I-IR neurons increase in number in brains from the second instar larval stage till the fifth instar larval stage. However, they decrease in number in the brain of the adult. Thereafter, the LK I-IR neurons again increase to 8 in adult brain. IL, instar larva; DP, day-old pupa.

procedures, very similar to those in the cryostat section immunostaining, can be described in brief as the followings: The whole brains were incubated with primary antiserum, anti-LK I, diluted to 1:1000 in phosphate buffered saline for 5 days at 4℃. Reaction with secondary and tertiary antisera (dilutions as above) was 1 day each and the peroxidation reaction was run for about 15min. The insect whole brains were dehydrated in graded alcohol concentrations and propylene oxide, and embedded in Durcupan (Fluka).

Results

Larval brains

The LK I-IR neuron begins to occur in the brain from the second instar larva (Fig. 1). The number of the LK I-IR neuron in the brain increases until the fifth instar larval stage, where the brain contains the largest number of the LK I-IR neurons in the larval stages examined in this experiment. However, the LK I-IR neuron decreases in number in the sixth instar larval brain.

The LK I-IR neuron was not found in first instar larval brain (Fig. 2a). In second instar larval brain, four LK I-IR cell bodies that shows bilateral symmetry in their location are shown in dorsal cortex of both cerebral hemispheres (Figs. 2b and 4a). As shown in Fig. 2b, they are located in dorsal cortex of posterior brain. However, the LK I-IR nerve processes could not be seen in the brain. The third instar larval brain contains a total of 15 LK I-IR neurons (Fig. 2c). The 9 LK I-IR cell bodies can be found in the cortex of anterior brain, while the 6 cell bodies are seen in the cortex of posterior brain. Some of the LK I-IR nerve fibers which are supposed to project from 2 LK I-IR cell bodies in dorsal cortex run down to middle and ventral parts of both central neuropils, as

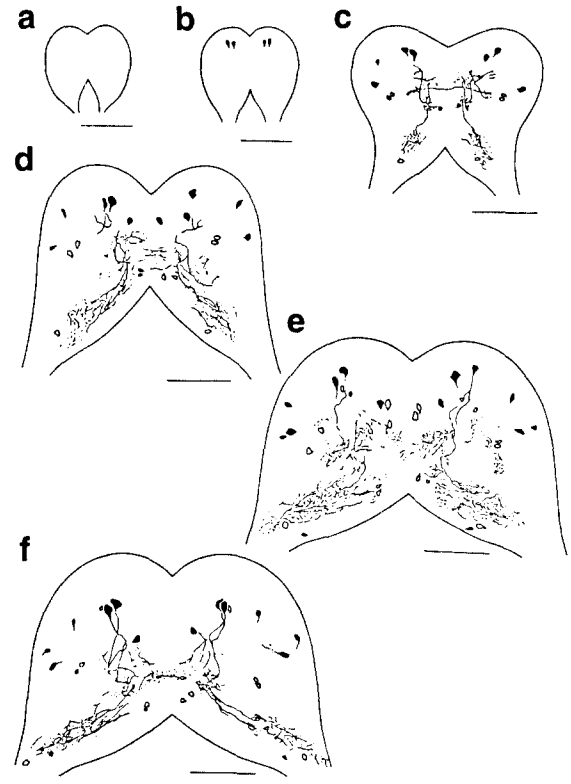

Fig. 2. Schematic diagram for localization of LK I-IR neurons in larval barins. Frontal and rear views from whole brains. Dark neurons imply those which are located in posterior parts of each brain, whereas empty neurons indicate those which are located in anterior parts. All scale bars=100 μm. (a) First instar larval brain. (b) Second instar larval brain. (c) Third instar larval brain. The processes derived from the LK I-IR neurons constitute a part of neural networks in central neuropils of each hemisphere. In particular, the axon (arrowhead) of the LK I-IR neuron runs dorsally to centralateral neuropil, forming the LK I-IR commissure. (d) Fourth instar larval brain. (e) Fifth instar larval brain. The LK I-IR cell bodies and processes continue to increase in number. A LK I-IR neuron (arrowhead) bifurcates and one of two axons runs down ventrally. (f) Sixth instar larval brain.

seen in Fig. 2c. In those regions, the LK I-IR nerve fibers show rich arborization. A commissural LK I-IR nerve fiber is located in the middle part between both central neuropils (Fig. 4b).

The fourth instar larval brain includes total 20 LK I-IR neurons (Figs. 2d and 5). There are 10 LK I-IR cell bodies in middle and ventral cortex of anterior brain, whereas 10 LK I-IR cell bodies are found in dorsal and lateral cortex of posterior brain. The LK I-IR nerve fibers which are found to project mainly from dorsal cortex of posterior brain run down into middle and ventral parts of both central neuropils. In addition, the commissural LK I-IR nerve fibers can be also seen in middle parts between both central neuropils. The 32 LK I-IR neurons are located in fifth instar larval brain (Figs. 2e and 6a, b). Most of the LK I-IR cell bodies are located in dorsal and lateral cortex of anterior and posterior brain, while a small number of the LK I-IR cell bodies are shown in ventral cortex of anterior and posterior brain. Some

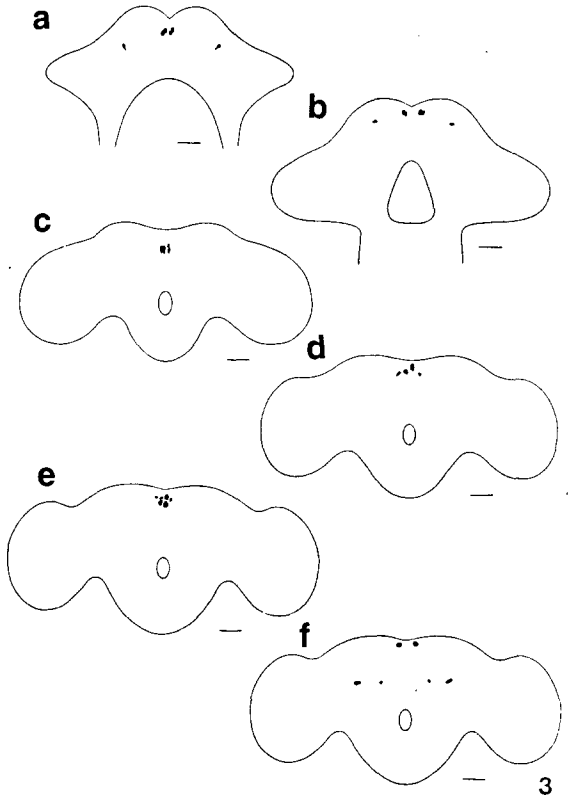

Fig. 3. Schematic mapping of the LK I-IR neurons in prepupal, pupal and adult brains. Frontal and rear views from whole brains. All scale bars = 100 μm. (a) Prepupal brain. The four LK I-IR neurons are localized in the pars intercerebralis(PI), whereas each one (empty neuron) is found in each dorsolateral region of the brain. (b) One-day-old pupal brain. Es, esophagus. (c) Three-day-old pupal brain. (d) Five-day-old pupal brain. (e) Seven-day-old pupal brain. (f) Adult brain.

of the LK I-IR cell bodies located in both dorsal cortex of posterior brain project their axons into both central neuropils. The LK I-IR nerve fibers projecting into both central neuropils show abundant arborization in middle and ventral parts of both central neuropils.

The sixth instar larval brain contains total 26 LK I-IR neurons (Figs. 2f and 7a, b, c). The LK I-IR cell bodies are seen in dorsal, lateral and ventral cortex of anterior and posterior brain. However, most of the

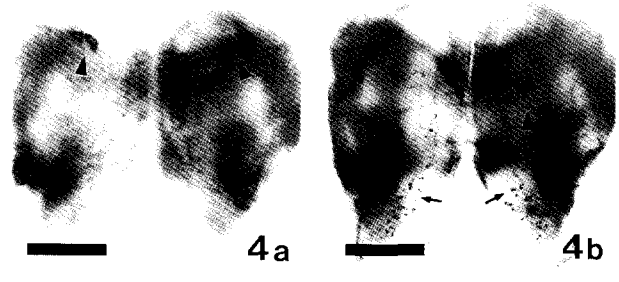

Fig. 4. Third instar larval brain. Scale bars = 50 μm. (a) Rear view from the whole brain. Two pairs of LK I-IR cell bodies (arrowheads) are found in dorsal cortex. (b) Frontal view from the whole brain. The LK I-IR commissural fibers (arrowhead) are located in middle of two cerebral hemispheres. The ventral neuropils of both hemispheres also include arborization (small arrows) of the LK I-IR nerve fibers which project from the LK I-IR cell bodies in dorsal cortex of the brain.

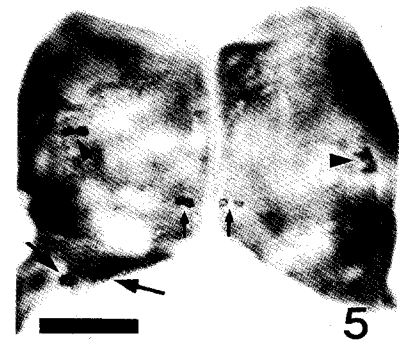

Fig. 5. Frontal view from fourth instar larval brain. Two pairs of the LK I-IR cell bodies (arrowheads) show bilateral location in middle cortex of lateral portions of the whole brain. Lower middle cortex of both hemispheres also includes two pairs of small LK I-IR cell bodies (small arrow). Scale bar = 50 μm.

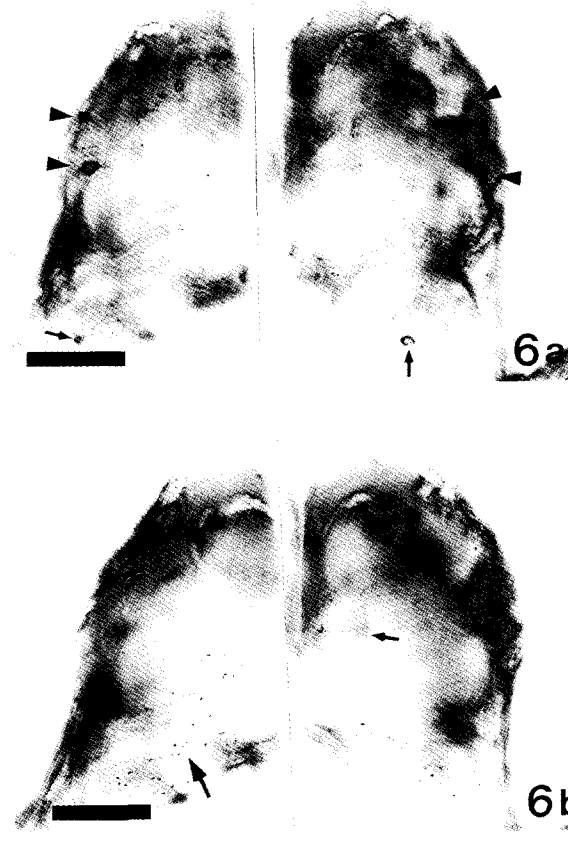

Fig. 6. Two photographs of fifth instar larval whole brain. All scale bars=50 μm. (a) The brain shows bilateral location of both two pairs of the LK I-IR cell bodies (arrowheads) in middle cortex of cerebral lateral portions, and one pair of those (small arrows) in cerebral ventral cortex near ventral nerve cord. (b) The left cerebral hemisphere contains a LK I-IR cell body (arrowhead) in dorsal cortex and nerve fiber (small arrow) which is thought to be derived from that cell body. The contralateral hemisphere includes varicose arborization (large arrow) of the LK I-IR nerve fibers.

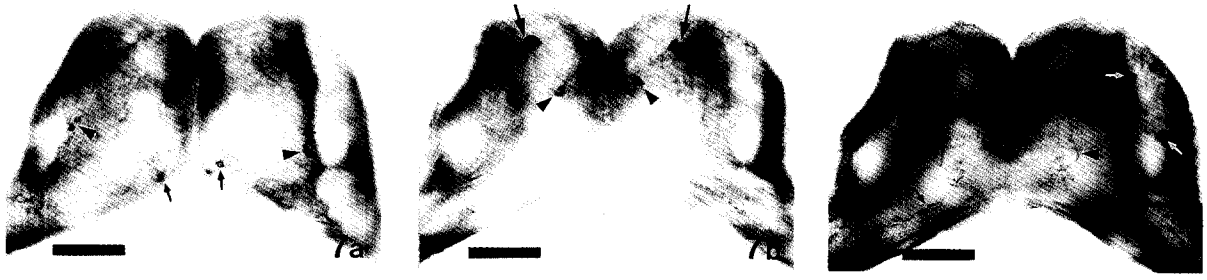

Fig. 7. Three photographs of sixth instar larval whole brain. Scale bar = 50 μm. (a) The brain includes two pairs of LK I-IR cell bodies (arrowheads) in anterolateral cortex and one pair of LK I-IR cell bodies (small arrows) near medioventral cortex. (b) The posterodorsal cortex of the brain contains two pairs of LK I-IR cell bodies (large arrows), while one pair of LK I-IR cell bodies (arrowheads) show bilateral symmetry in the location in cortex near pars intercerebralis. (c) Middle part of sixth instar larval brain includes the LK I-IR cell bodies (small white arrows) and nerve fibers (arrowhead and small dark arrows). A LK I-IR nerve fiber in left hemisphere is running down to ventral part of neuropil. A pair of LK I-IR nerve fibers (small dark arrows) are passing down to ventral nerve cord through ventral neuropil.

LK I-IR cell bodies located in dorsal and lateral cortex were found in posterior brain, whereas most of the LK I-IR cell bodies located in ventral cortex shown in anterior brain. The obvious projections of the LK I-IR nerve fibers from dorsal cortex of posterior brain are easily found. Those LK I-IR nerve fibers run down into middle and ventral parts of both central neuropils. Of course, the commissural LK I-IR nerve fibers could be shown in the middle between central neuropils of both sides.

Prepupal brain

The LK I-IR neurons abruptly decrease to 6 in number in the prepupal brain (Fig. 3a). The reactivities of the LK I-IR neurons to the antibody became weak. Only the cell bodies in the neurons reacted to the antibody. Therefore, the LK I-IR nerve process could not be found in the prepupal brain. The 4 of 6 LK I-IR cell bodies are located in pars intercerebralis of posterior brain, whereas 2 are found in the lateral cortex of anterior brain.

Pupal brains

The LK I-IR neurons showed weak reactivities to the LK I antibody. Therefore, the LK I-IR cell bodies could be immunostained, while their nerve processes were not found in all the pupal brains examined in this experiment.

The one-day-old pupal brain has a total of 6 LK I-IR neurons (Fig. 3b). Two LK I-IR cell bodies are located in the calyx of anterior protocerebrum, whereas 4 are found in the pars intercerebralis of posterior brain. The three-day-old pupal brain includes about 4 LK I-IR neurons of which cell bodies can be found in the pars intercerebralis of posterior brain (Fig. 3c). The 4 LK I-IR cell bodies are clustered in a group in the pars intercerebralis. In the five-day-old pupal brain, there are 4 LK I-IR neurons (Fig. 3d). Their cell bodies are slightly scattered in the pars intercerebralis of posterior protocerebrum (Fig. 8). The seven-day-old pupal brain contains 6 LK I-IR neurons (Fig. 3e). All the cell bodies of six LK I-IR neurons are grouped in the pars intercerebralis of the posterior brain.

Adult brain

Adult brain also showed weak reactivities of the LK

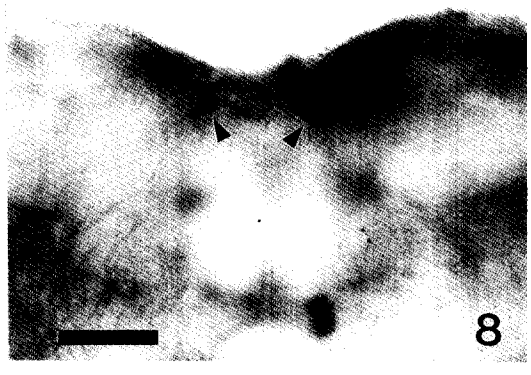

Fig. 8. A pair of LK I-IR cell bodies (arrowheads) showing bilateral location in pars intercerebralis of fifth instar larval whole brain. Scale bar=25 μm.

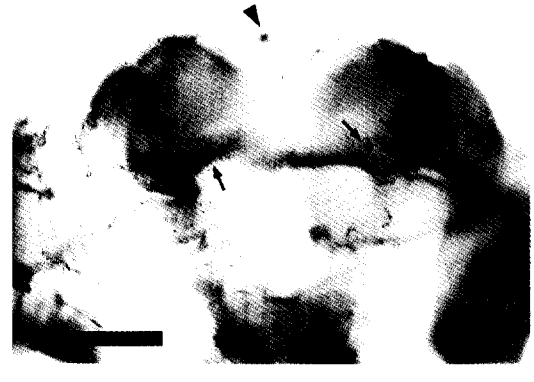

Fig. 9. LK I-IR neurons in adult whole brain. The LK I-IR cell bodies are located in pars intercerebralis (arrowhead) and middle part of posterior cortex (arrows). Scale bar=50 μm.

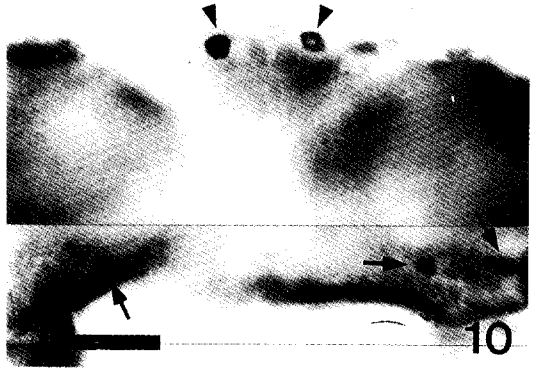

Fig. 10. Higher magnification of the LK I-IR cell bodies seen in pars intercerebralis (arrowheads) and middle part of the posterior cortex (arrows) of whole brain in figure 9. Scale bar=25 μm.

I-IR neurons to the antibody. The LK I-IR neurons slightly increased to 8 in number (Fig. 3f). As shown in pupal brains, the nerve processes of the LK I-IR neuron could not be found in all areas of the adult brain. However, the LK I-IR cell bodies were found (Figs. 9 and 10). The two large LK I-IR cell bodies were located in the pars intercerebralis of the posterior brain (Figs. 9 and 10). The remaining 6 LK I-IR cell bodies were seen in the middle cortex of the posterior mushroom body.

Discussion

Detailed description of LK I-IR neurons in adult brain

Adult brain of the common cutworm *Spodoptera litura* used in this experiment contains totally, 8 LK I-IR neurons. The localization of the LK I-IR neurons is confined to the pars intercerebralis and the middle cortex of posterior mushroom body. However, in the brains of the cockroach *Leucophaea maderae* (Nässel et al., 1992), locust *Locusta migratoria* (Nässel, 1993b), cockroach *Nauphoeta cinerea* (Chen et al., 1994a), larger number of the LK I-IR neurons were described. Nässel et al. (1992) mentioned about 160 LK I-IR neurons in the protocerebrum and optic lobes. A total of about 140 leucokinin-like immunoreactive(LK-LI) neurons were also shown to be resolved in the brain of the locust *Locusta migratoria* (Nässel, 1993b). Each hemisphere contains 23 LK-LI cell bodies in the optic lobe, 30 in anterior protocerebrum, 9 in the posterior protocerebrum, and 6 in the tritocerebrum. In the cockroach *Nauphoeta cinerea*, 130-155 LK IV-IR neurons and neurosecretory cells were demonstrated to be located in pars intercerebralis, pars lateralis, protocerebrum, and optic lobe of the brain (Chen et al., 1994a). Concerned with number and location of the leucokinin-immunoreactive (LK-IR) neurons in the brain of four insects, experimental results suggest that LK-IR neurons obtained from *Spodoptera* are remakably different from those of *Leucophaea*, *Locusta* and *Naupheta*. In particular, adult brain of *Spodoptera* contains smaller number of LK-IR neurons than those in adult brains of *Leucophaea*, *Locusta* and *Naupheta*. In addition, 8 LK I-IR nerve processes in adult brain of *Spodoptera* could be not found, because the LK I-IR neurons show very weak reactivities to the antisera used in this experiment. Concerned with the findings of LK I-IR neurons in adult brain of *Leucophaea*, Nässel et al. (1992) mentioned that *Leucophaea* brain might contain both neuropeptides of leucokinin type and peptides resembling some of the vertebrate tachkinins, and it is also not to be excluded that the leucokinin I antisera cross-reacted with the other seven known leucokinins of *Leucophaea*.

Postembryonic developmental aspects of LK I-IR neurons

The LK I-IR neurons are found at the larval stages, pupal stages, and adult stage. The first immunoreactivity in the brain occurs during second instar larva. The number of the LK I-IR neurons in the brain increase from the second instar larva to the fifth instar larva. The brain of the fifth instar larva has about 32 LK I-IR neurons, suggesting the largest number in all the postembryonic stages in *Spodoptera*. Thereafter, the number of the LK I-IR neurons begin to decrease. During the pupal stages, the brains continue to retain 4 or 6 LK I-IR neurons. Adult brain includes slightly increased number (about 8 LK I-IR neurons). Interestingly, when the LK I-IR neurons first appear in second instar larva, they show a high level of immunoreactivity. The higher intensity of their immunoreactivity persists until the sixth instar larval stage. However, the strong immunoreactivities of the LK I-IR neurons in the larval stages began to chang from one-day-old pupa which begins to display weak immunoreactivity. According to description of Cantera et al. (1994), postembryonic development of corazonin-containing neurons and neurosecretory cells in the blowfly *Phormia terraenovae*, the gradual acquisition of immunoreactivity by the LK I-IR neurons in the brain can be interpreted as reflecting the progress of the differentiation of an imaginal type. In other words, either these cells are recruited from a larval subset of non-immunoreactive neurons, or they are the cells formed by postembryonic proliferation.

Some of the LK I-IR neurons in the brain of the fifth instar larva begin to lose their immunoreactivities. These phenomena which lose immunoreactivities in the LK I-IR neurons of the brains from the fifth instar larva are more remarkable during metamorphic change to the adult. Despite most, if not all, peptidergic and monoaminergic neurons are known to persist through metamorphosis with retained immuno-

reactivity (Cantera and Nässel, 1987, 1992; Nässel et al., 1988; Breidbach and Dricksen, 1989; Truman, 1990; Davis et al., 1993; Zitnan et al., 1993), it is not clear whether the disappearance of LK I -immunoreactivity is due to death of the neurons or a switch in synthesis of neuroactive molecules in surviving neurons. An example of changing neuropeptide phenotype in given neurons during metamorphosis has been demonstrated in abdominal ganglia of the tobacco hawkmoth *Manduca sexta* (Tublitz and Sylwester, 1990). However, Cantera et al. (1994) mentioned that it is unlikely that the immunoreactive peptide of the corazonin-immunoreactive neurons in the blowfly (*Phormia terraenovae*) thoracic and abdominal neuromeres become redistributed from the cell bodies to the neuron processes during metamorphosis. Further studies concerning the loss of the LK-immunoreactivity in the LK I-IR neurons in the *Spodoptera* brain is necessary.

Acknowledgements

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