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Postembryonic Changes of Locustatachykinin I-immunoreactive Neurons in the Brains of the Moth *Spodoptera litura*

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The antiserum against locustatachykinin I, originally isolated from brain and retrocerebral complex of the locust *Locusta migratoria*, has been used to investigate changes in number, localization, and structure of locustatachykinin I-immunoreactive (LomTK I-IR) neurons in the brains of the common cutworm, *Spodoptera litura*, during postembryonic development. These neurons are found at larval, pupal, and adult stages. In the larval stages, the first instar larva shows the first appearance of about 8 LomTK I-IR neurons. These neurons gradually increase in number from the second to fourth instar larvae which have the largest number of about 92 in all postembryonic stages. Thereafter, these neurons decrease to about 28 in number in the 5-day-old pupa. However, they begin to rise again from the 7-day-old pupal stage, eventually reaching to about 90 in the 1-day-old adult. The developing larval brains contain cell bodies of these neurons in most neuromeres. After the metamorphosis of larva to pupa and adult, localization of these neuronal cell bodies is confined to the specific cerebral neuromeres. The 7-day-old pupal brain shows the location of these neuronal cell bodies in pars intercerebralis, pars lateralis of protocerebrum, deutocerebrum, tritocerebrum, optic lobe-near region, and subesophageal ganglion. In the 1-day-old adult, however, the brain has these cell bodies only in some neuromeres of protocerebrum, deutocerebrum, and subesophageal ganglion. Throughout the postembryonic life, changes in structure of these neurons coincide with changes in number and localization of these neurons. These findings suggest that changes in number, localization, and structure of these neurons reflect differentiation of these neurons to adult type.

The tachykinins are a large peptide family that has been isolated from some vertebrates (Nässel, 1993a). It has been demonstrated that the tachykinins are also produced as neurotransmitters in various insects such as *Leucophaea maderae* (Hansen et al. 1982), *Drosophila melanogaster* (Nässel et al., 1992), *Calliphora vomitoria* (Lundquist et al., 1993; Nässel et al., 1995), *Locusta migratoria* (Nässel, 1993b), and *Manduca sexta* (Chen et al., 1994). The insect tachykinins consist of several neuropeptides: substance P, callitachykinin (Nässel et al., 1995), and locustatachykinins (Blitz et al., 1995). One of the well-known insect tachykinins is substance P. It has been demonstrated in pharmacological studies that most of insect tachykinins have myotropic effects on intestine and oviduct of *Locusta migratoria* (Schoofs et al., 1990a, b).

Four kinds of locustatachykinins designated LomTK I, LomTK II, LomTK III, and LomTK IV were originally isolated from brain and retrocerebral complex of *Locusta migratoria* (Schoofs et al., 1990a, b). They stimulate the contraction of visceral muscle in *Calliphora vomitoria* (Lundquist et al., 1994). These four locustatachykinins show some homologies of amino acid sequence with the vertebrate tachykinins (Schoofs et al., 1990a, b). In particular, the LomTK I has amino acid sequence of Gly-Pro-Ser-Gly-Phe-Tyr-Gly-Val-Arg-amide (Lundquist et al., 1994; Nässel et al., 1995).

LomTK I-IR neurons have been recently described in the nervous system of several adult insects, using anti-LomTK I antiserum which recognizes C-terminus of the LomTK I neuropeptide sequence (Nässel, 1993a, b, and 1995; Lundquist et al., 1994; Muren et al., 1995; Nässel et al., 1995; Blitz et al., 1995). A total of about 160 neurons has been found in the adult brain of the blowfly *Calliphora vomitoria* (Lundquist et al., 1994). The axonal projection from these neuronal cell bodies could be traced in many regions of both central neuropils, except in the mushroom body and optic lobe. Most of these neurons in blowfly brain were interneurons, suggesting that this neuropeptide may

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play an important role as a neurotransmitter in the brain. However, it has also been shown that this neuropeptide has action as a neurohormone in *Locusta migratoria* (Nässel et al., 1995). This is involved in the release of adipokinetic hormone from corpora cardiaca of *Locusta migratoria*.

A detailed mapping of peptide-producing neurons and neural networks organized by these neurons are useful as a starting point when searching for release sites and functional roles of different peptide messengers in the nervous system. Investigations on changes in number, localization and structure of peptide-producing neurons, such as LomTK I-IR neurons, in the insect brain during postembryonic development may give information on functional roles of various peptides at different developmental stages. However, this kind of investigation is described only on a few specific neurons including corazonin-containing neurons and neurosecretory cells in *Phormia terraenovae* (Cantera et al., 1994) and leucokinin-like immunoreactive (LK-LI) neurons in *Spodoptera litura* (Kang and Lee, 1997). The objective of this present investigation is to clarify morphological differentiation of LomTK I-IR neurons in the postembryonic brain of the common cutworm *Spodoptera litura*, using anti-LomTK I antiserum.

Materials and Methods

Experimental insects

A colony of the commom cutworm *S. litura* were maintained under a constant photoperiod (16 Light and 8 Dark cycle) at about 70% relative humidity and 25℃ in a large growth chamber. They were reared on artificial diet syrup. The first, second, third, fourth, fifth and sixth instar larvae, and prepupae, one-, three-, five- and seven-day-old pupae, and one-day-old adults were selected, and more than 10 individuals were used as experimental insects at each developmental stage.

Immunocytochemistry

The anti-locustatachykinin I (anti-LomTK I) used as a primary antibody was kindly provided by Dr. Dick R. Nässel (Stockholm University, Sweden). Details on antiserum production and cross reactivity are given by Nässel et al. (1992), Muren et al. (1993), and Winther et al. (1996).

Immunocytochemistry was performed with the peroxidase anti-peroxidase (PAP) method slightly modified from that of Cantera and Nässel (1992). The head cuticles of larvae, pupae, and adults at each postembryonic developmental stage mentioned above were opened by dissection in 0.1 M phosphate buffer (pH 7.4) at 4℃, and whole brains were completely isolated from the heads. The isolated brains were then fixed by immediate immersion into 4% paraformaldehyde in phosphate buffer for 12 to 16 h. Thereafter, the fixed brains were thoroughly rinsed several times with 0.1 M phosphate buffer for 2 h at room temperature.

For the wholemount immunostaining, the brains were immersed in primary antiserum (anti-LomTK I) diluted to dilution buffer (DiB ; consisting of 0.01 M phosphate buffered saline with 0.25% Triton X-100) to 1 : 1000 for 6 to 10 h at 4℃. After thorough washes in wash buffer (0.1 M phosphate buffer containing 0.25% Triton X-100), unlabeled swine anti-rabbit immunoglobulin (Dako) diluted in DiB to 1 : 50 was used as a bridge for 24 h at 4℃, and rabbit peroxidase-anti-peroxidase (DAKO) at 1 : 100 was applied as a third layer for 24 h at 4℃. Diaminobenzidine (0.03% in Tris-HCl with 0.01% H_2O_2 at pH 7.2; Sigma) was used as a chromogen. The brain tissues were dehydrated and then embedded either in Durcupan (Fluka) or glycerin for the detailed drawings. The stained neurons with anti-LomTK I were examined and photographed with a Nikon Optiphot-2 microscope. Number of the neurons within a brain were counted by drawings of these neurons made with a camera lucida attachment.

Adult brains were also used for cryostat sectioning. The fixed brains were thoroughly rinsed several times in 0.1 M phosphate buffer and immersed into 20% sucrose in 0.1 M phosphate buffer overnight at 4℃. Cryostat sections were made at -14℃ with 25 μm thickness (Leitz Cryostat). The remaining procedures were described elsewhere (Kang and Lee, 1997).

Results

Number of LomTK-IR neurons in the postembryonic brains

About 8 LomTK-IR neurons appeared first in the brain of the first instar larva (Fig. 1). In the second instar larva, these neurons increased gradually to about 53. This increasing trend in number of these neurons in the larval brains continued to the fourth instar larva which had about 92 in number. Thereafter, these neurons decreased from the fifth instar larva to the 5-day-old pupa which had about 28. In the 7-day-old pupa which

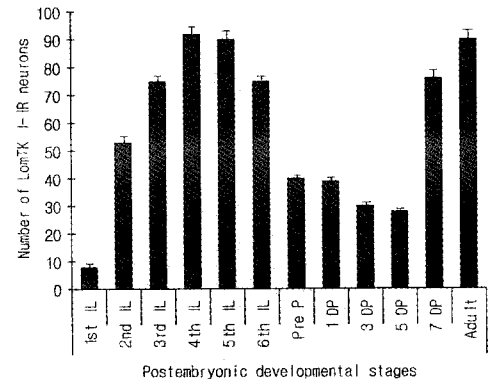

Fig. 1. Numerical changes of the LomTK I-IR neurons in brains at various postembryonic developmental stages of *S. litura*. Note that the number of these neurons becomes increased in both late larval and adult stages. IL, instar larva; DP, day-old pupa.

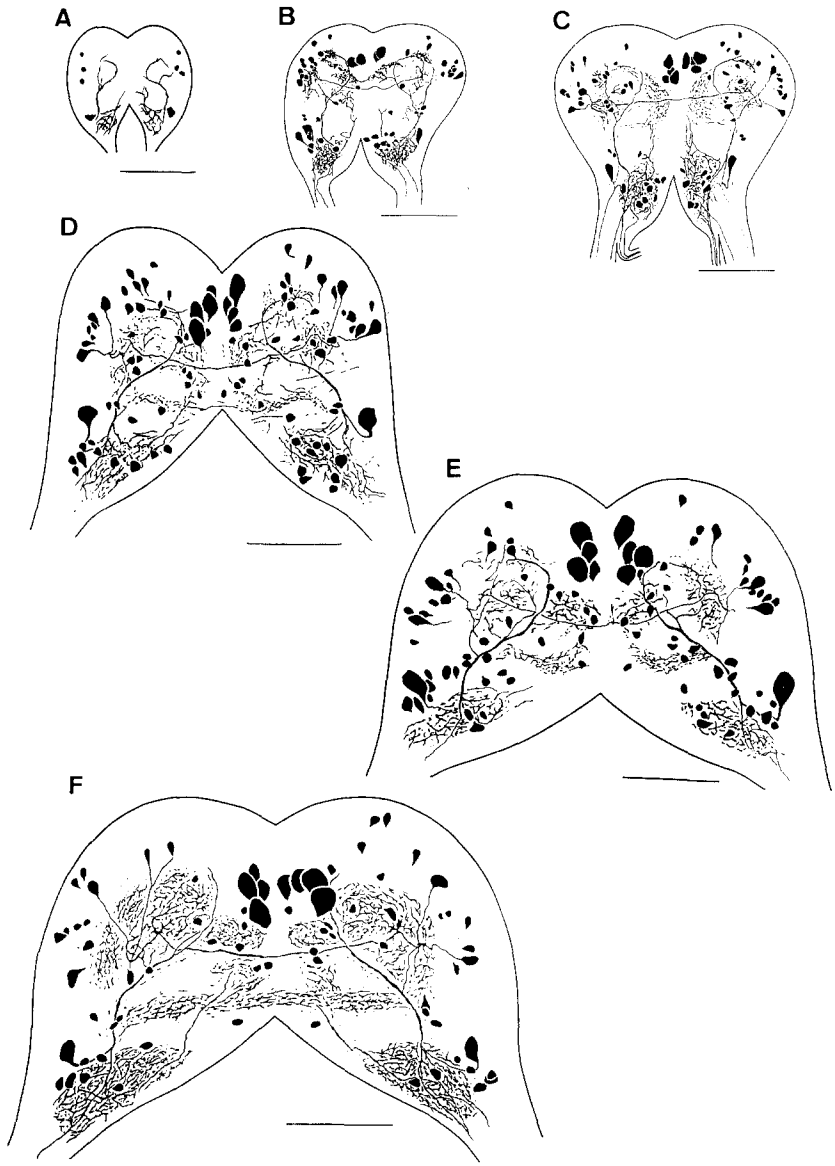

Fig. 2. Schematic diagrams of frontal views from a whole brain showing localization of LomTK I-IR neurons in the larval brains. (A) First instar larval brain. (B) Second instar larval brain. (C) Third instar larval brain. (D) Fourth instar larval brain. (E) Fifth instar larval brain. (F) Sixth instar larval brain. Scale bars=100 μm.

contained about 76, the number of these neurons again began to rise and then reached about 90 in number in the 1-day-old adult.

Larval brains

The first instar larval brain had about 6 LomTK I-IR cell bodies in the cortex of lateromiddle region and about 2 in the cortex of lateroventral region, respectively. These cell bodies located in the cortex of lateromiddle region were generally smaller in size than those in the cortex of ventrolateral region (Figs. 2A, 3B). These neurons occurring in the first instar larval brain were mostly interneurons, innervating several neuromeres of ipsilateral cerebral hemispheres. One cell body in the ventrolateral region projected its axon into a dorsal neuromere of central neuropil. This fiber bifurcated into two branches on its way to the dorsal neuropil. The ventral neuropils included abundant LomTK I-IR processes. The second instar larva had about 53 LomTK I-IR cell bodies throughout cerebral neuromeres. However, location of most of these cell bodies was concentrated on pars intercerebralis, dorsolateral and ventrolateral cortexes (Fig. 2B). The cell bodies localized in pars intercerebralis were larger than those in other neuromeres (Fig. 3B). As shown in Figure 2B, the LomTK

I-IR fiber formed a cerebral commissure between dorsal neuromeres of both central neuropils. One cell body located in the cortex of lateroventral neuromere projected an axon which bifurcated into two branches (Fig. 3B1). One of two branches ran up dorsally, whereas the other separated into two branches that ran to subesophageal ganglion and hypocerebral nerve connective, respectively. The ventral neuromeres of both central neuropils which showed the richest ramification was innervated by these fibers running up from both ventral connectives (Fig. 2B). The third instar larval brain which had about 75 LomTK I-IR neurons showed localization of the processes similar to those in the second instar larval brain (Figs. 2C and 3C). In the third instar larva, however, the brain, including afferent and efferent LomTK I-IR fibers, had larger numbers of these cell bodies and richer ramification of these processes than those in the second instar larval brain.

The fourth instar larval brain, containing about 92 LomTK I-IR neurons, had these cell bodies and processes in all the neuromeres (Fig. 2D). Accompanying with the increase in brain size in this developmental stage, cell bodies became larger in size than those in previous stages. The LomTK I-IR commissure rose to two in number (Fig. 2D). As shown in Fig. 3D, two fiber bundles projected from these cell bodies in pars intercerebralis decussated in a median neuromere with each other. Thereafter, they projected down into the contralateral neuropils, respectively.

In the fifth instar which had about 90 LomTK I-IR neurons in the brain of larva, the basic structure of these neurons was similar to that in the fourth instar larva (Fig. 2E). The LomTK I-IR commissure was maintained also in the fifth instar larval brain (Fig. 3E). The sixth instar larval brain which had about 75 LomTK I-IR neurons contained the most abundant arborization of these neurons in all the neuromeres (Fig. 2F), including these commissural fibers as shown in Fig. 3F.

Prepupal brain

The prepupal brain had about 40 LomTK I-IR neurons. The numerical and structural characteristics of these neurons in pars intercerebralis were found unchanged (Fig. 4A). The processes of LomTK I-IR median neurosecretory cells were revealed more conspicuously. However, these neurons in other neuromeres except pars intercerebralis were reduced in number and these processes also decreased in all the cerebral neuromeres.

Pupal brains

The 1-day-old pupal brain, containing about 39 LomTK I-IR neurons, had an increased number of these fibers, especially in a differentiating mushroom body (Figs. 4A and 5A). As shown in Figure 4B, a LomTK I-IR cell body located near optic lobe supplied its axon into differentiating alpha-lobe of mushroom body. The other cell bodies in the tritocerebral cortex projected their axons into differenciating alpha-lobe. On an entry to alpha-lobe, these fibers bifurcated to first two branches. One branch again separated into two second branches which ran up to the calyx of the mushroom body, whereas the other branch also separated into second two branches. One of them crossed the suboesophageal neuropil immediately beneath esophageal foramen innervating to the controlateral subesophageal neuropil, while the other branch ran down through subesophageal neuropil to ventral nerve connective.

The 3-day-old pupal brain, containing about 30 LomTK I-IR neurons, had more simply changed structure of these processes (Figs. 4C and 5C). However, the subesophageal neuropil contained abundant process than that in the 1-day-old pupa. The 5-day-old pupal brain in which a fan-shaped central body complex had rich LomTK I-IR processes contained about 28 LomTK I-IR neurons (Figs. 4D and 5E). The 7-day-old pupal brain, containing about 76 neurons (Fig. 4E), had about 10 cell bodies filled up with fine granules in pars intercerebralis (Figs. 6B-D). In addition to those in pars intercerebralis, these cell bodies found in other neuromeres became increased in number to about 8 in pars lateralis of the protocerebrum, about 16 in central body complex, about 14 in deutocerebrum, about 12 in tritocerebrum, about 6 near optic lobe, and about 10 in subesophageal ganglion, respectively (Figs. 6A, C, E, F, G, and H). Some of these cell bodies in the subesophageal ganglion projected their axons into its neuropil. As shown in Figures 6A and 6C, these cell bodies in the deutocerebrum projected their axons into the neuropil of antennal lobe.

Adult brain

Although LomTK I-IR cell bodies were dispersed in all the neuromeres of the 1-day-old adult brain, most of them were concentrated mostly on pars intercerebralis, antennal lobe and subesophageal ganglion (Figs. 7A and B). The brain had these fibers which connected the protocerebrum with the optic lobe (Fig. 5D). The basic structure of LomTK I-IR median neurosecretory system also remained unchanged in adult brain (Figs. 5F and 7B). The cell bodies near the optic lobe projected their axons into lateral protocerebrum (Figs. 6F and G). In addtion, all antennal glomeruli showed varicosities from these fibers (Figs. 6B, C, and E). As shown in Fig. 7A, some of these ramifications were derived from cell bodies located near antennal lobe. The subesophageal neuropil also contained rich ramification of these fibers (Fig. 5B).

Discussion

The adult brain of *S. litura* used in this investigation has about 90 LomTK I-IR neurons. These neuronal cell bodies are mostly localized in pars intercerebralis, deu-

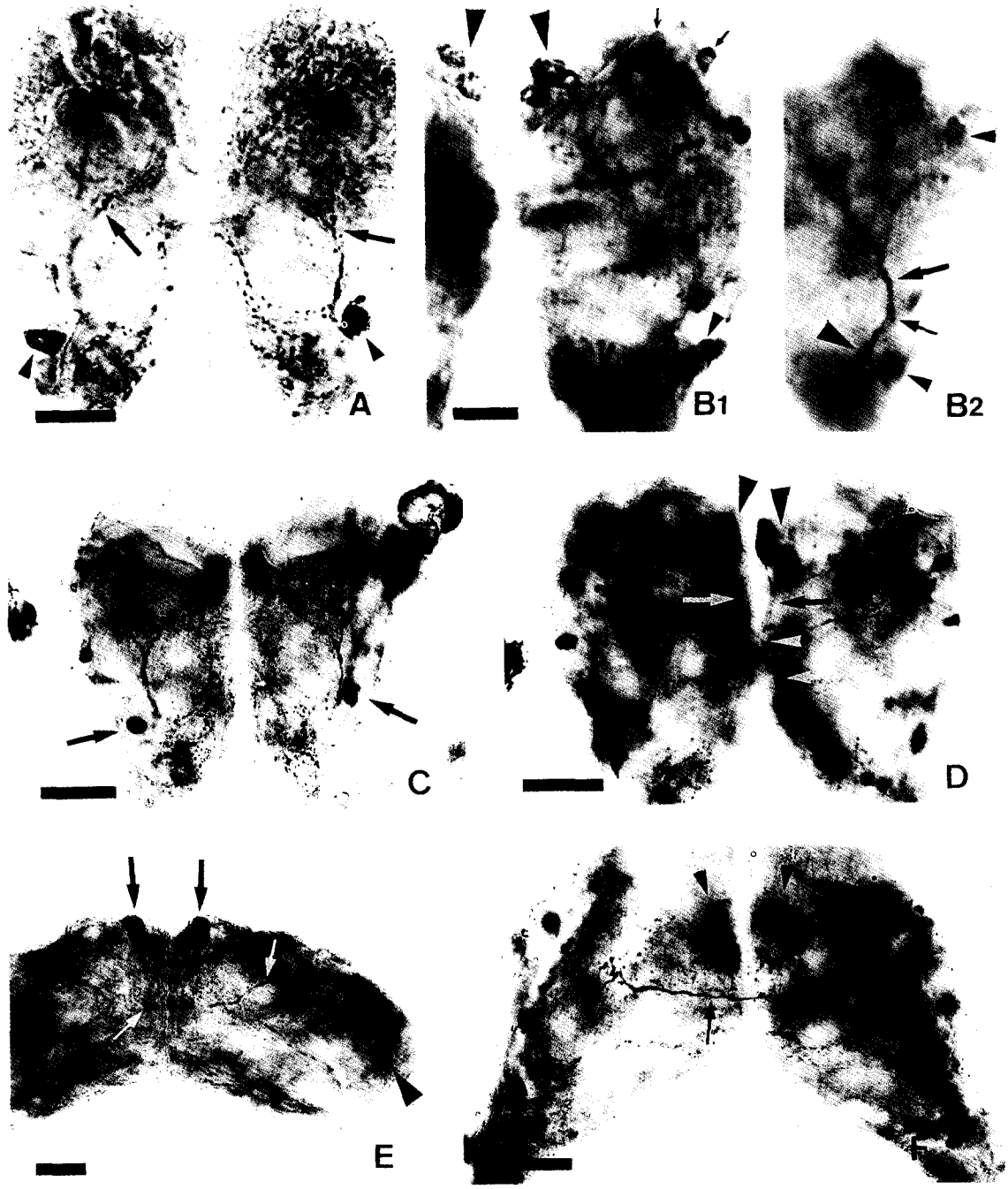

Fig. 3. Photographs of localization of LomTK I-IR neurons in larval whole brains. (A) Frontal view from the first instar larval brain. One pair of cell bodies (arrowheads) are located in the ventral region of each hemisphere. One pair of fibers derived from cell bodies (arrowheads) run up dorsally through each central neuropil. (B1) Frontal view from the second instar larval brain. The eight (large arrowhead) and a few of cell bodies (small arrows) are found in pars intercerebralis and celebral lateral region. The big cell bodies (small arrowhead) are located in the same region as those (arrowheads in Fig. A) found in the first instar larval brain. (B2) Frontal view from the second instar larval brain. A cell body (small arrowhead), the same as those in ventral region in Fig. B1 projects an axon (small arrowhead) which bifurcates into two axons: one axon runs up dorsally (large arrow) and another one again bifurcates into two branches (large arrow) that run for suboesophageal ganglion and hypocerebral nerve connective, respectively. Magnification of this photograph is the same as that of Fig. B1. (C) Frontal view from the third instar larval brain. This brain shows a symmetrical location of cell bodies (arrows). These neurons are continuously located in the same position as from the first instar larval brain to adult brain. (D) Frontal view from the fourth instar larval brain. Four pairs of neurons (two arrowheads) project their axons (arrows) into ventral, median neuropil, in which the axons cross (small arrowhead), and then run to controlateral corpora cardiaca. (E) Horizontal view from the fifth instar larva. Four pairs of cell bodies (two black arrows) are strongly labeled in anterior pars intercerebralis. The commissural fiber (white arrows) is also seen. A cell body (arrowhead) belongs to a cell body group in ventral region in Figs. A, B1, B2, and C. (F) Frontal view from the sixth instar larval brain. Four pairs of cell bodies (two arrowheads) are found in median part. The commissural neurons supply their axons (arrow) to controlateral neuropils. Scale bars=20 μm (B1), 25 μm (A), and 50 μm (C, D, E, and F).

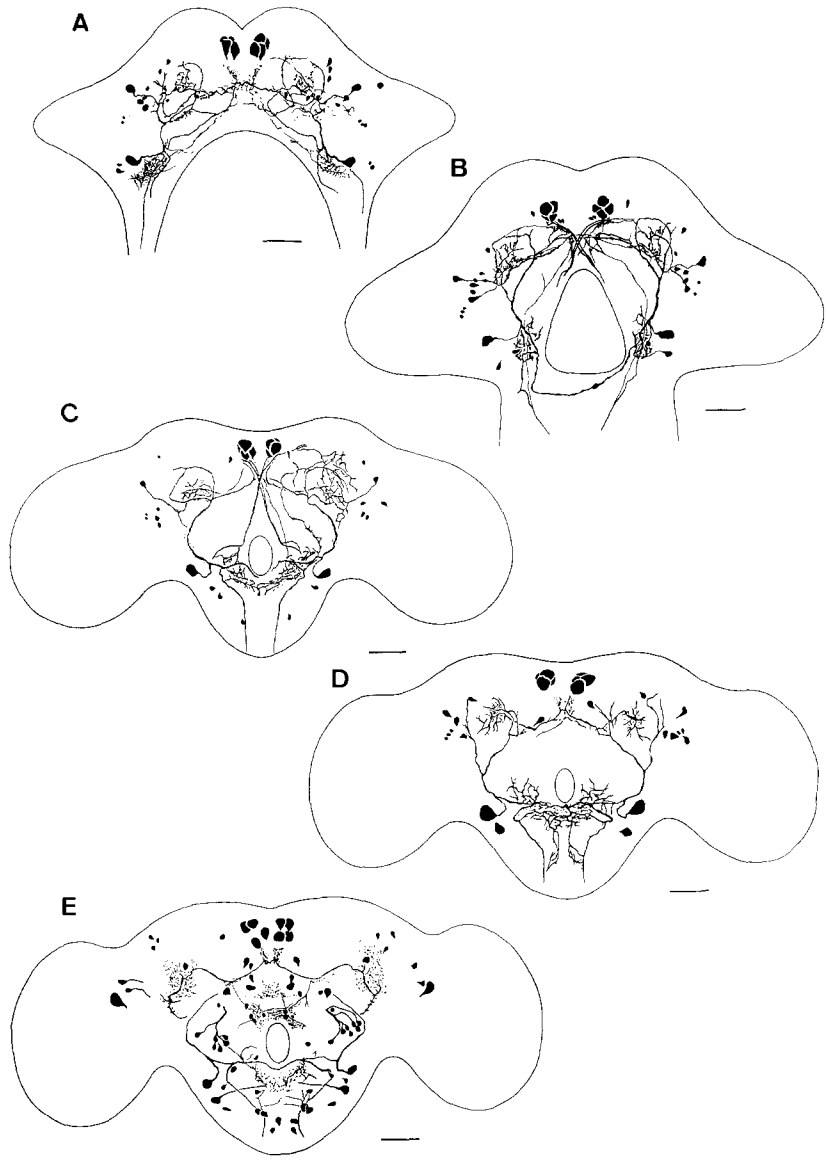

Fig. 4. Tracings of LomTK I-IR neurons in the prepupal and pupal brains. Frontal views from whole brains. (A) Prepupal brain. (B) One-day-old pupal brain. (C) Three-day-old pupal brain. (D) Five-day-old pupal brain. (E) Seven-day-old pupal brain. Scale bars=100 μm.

tocerebrum, and subesophageal ganglion. The fibers, derived from the cell bodies in pars intercerebralis decussate in the median neuropil, and then appear to project into the retrocerebral complex consisting of corpora cardiaca and corpora allata. The remaining processes can be found in the lateral protocerebrum, antennal lobe and subesophageal neuropil. Therefore, adult brains of *S. litura* contain a smaller number of these neurons than those in the brain of *Locust migratoria* in which approximately 800 LomTK-LI neurons are found in the proto-, deuto- and tritocerebra, and optic lobe (Nässel, 1993b).

Processes of these neurons in *Locusta migratoria* innervate most of the synaptic neuropils of the brain and optic lobe (Nässel, 1993b). In the brian of *Calliphora vomitoria*, however, a total of about 160 LomTK I-IR neurons was described in the proto-, deuto-, and tritocerebra and subesophageal ganglion (Lundquist et al., 1994). It has been also demonstrated in *Calliphora vomitoria* that the processes derived from these neurons can be traced in various cerebral neuromeres, such as superior and dorsomedian regions of protocerebrum, optic tubercle, fan-shaped body and ventral bodies of the central complex, all glomeruli of the antennal lobes, and tirtocerebral and subesophageal neuropil. Compared results obtained from *S. litura* with those from *Locusta migratoria* and *Calliphora vomitoria*, the processes of these neurons in three

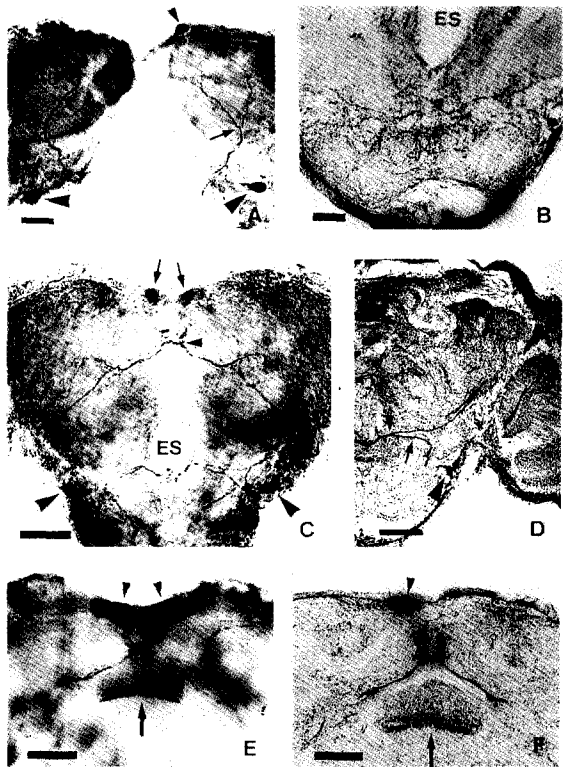

Fig. 5. Photographs of localization of LomTK-IR neurons in one-, three- and five-day-old pupal whole brains and cryocut adult brain. (A) Frontal view from one-day-old pupal brain. A cell body (small arrowhead) is seen in pars intercerebralis. The axons of bilateral cell bodies (two large arrowheads) below the glomeruli of antennal lobe (AL) run up dorsally and then bifurcate (arrow) to two branches. (B) Suboesophageal ganglion incorporated into cryocut and immunostained adult. Es, esophagus. (C) Frontal view from three-day-old pupal brain. The axons of these neurons (two arrows) runs posteroventrally and then cross (small arrow) in median regions. The axons of bilateral these neurons (large arrow) bifurcate into two branches. (D) Cryocut and immunostained adult brain. The axon (arrow) projected from a cell body (arrowhead) bifurcates to two branches: one branch runs up dorsally for corpus peduculatum and the other transverse to controlateral neuropil of suboesophageal ganglion. (E) Front view from five-day-old pupal whole brain. These cell bodies (two arrowheads) are localized in the pars intercerebralis. The central body shows LomTK I-immunoreactivities (arrow). (F) Cryocut and immunostained adult brain. Scale bars=50 μm (A, B) and 100 μm (C, D, E, F).

insect brains are innervated commonly to the antennal lobe and subesophageal ganglion. In addition, great differences in number, localization, and innervation of these neurons in adult brains of above three insects lead to ask for further progresses in researches on the genetic level of these neruons and on their more specific roles.

Developmental aspects of LomTK I-IR neurons in postembryonic brains

The number, localization and structure of these neurons in the brain of *S. litura* are changed during postembryonic development, as shown in Figs. 1, 2, and 4. During postembryonic development, the number of these neurons increases greatly in the brains of the fourth instar larva and the adult. This suggests that LomTK I-IR

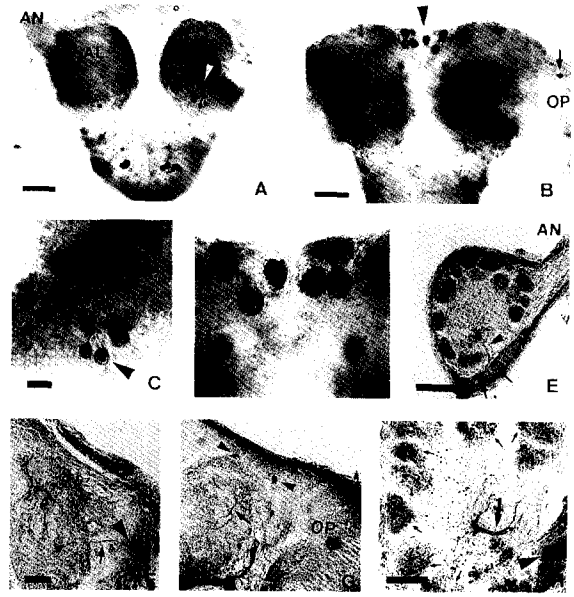

Fig. 6. Photographs of localization of LomTK-IR neurons in both seven-day-old pupal whole brain and cryocut and immunostained adult brain. (A) Anterior view from seven-day-old pupal whole brain. Two groups of these cell bodies (white arrowhead) are located in each antennal lobe. AL, antennal lobe ; AN, antennal nerve. (B) Posterior view from seven-day-old whole brain. In posterior part of the pars intercerebralis four pairs of large cell bodies (arrowhead) are located. A small neuron (arrow) is found in optic lobe. (C) Higher magnification of the neurons in left antennal lobe of Fig. 6A. The axons (arrow) are projected into the glomeruli of the antennal lobe from these cell bodies (arrowhead). (D) Higher magnification of these cell bodies in posterior part of the pars intercerebralis of Fig. 6B. (E) The antennal lobe of the adult brain cryocut and immunostained. Some of rich LomTK I3-IR varicosities in glomeruli are formed from the axons (arrowhead) of these cell bodies (arrow). AN, antennal nerve. (F) Cryocut and immunostained adult brain. A cell body (arrowhead) projects its axon (arrow) into lateral protocerebrum. This neuron is same as that (arrow) in the optic lobe of Fig. 6A. (G) Cryocut and immunostained adult brain. These cell bodies (arrowheads) are found in cortex of lateral protocerebrum which includes some axon (arrow) and its ramification. OP, optic lobe. (H) Higher magnification of antennal lobe of cryocut and immunostained adult brain. All glomeruli (small arrows) are filled with these processes. The antennal lobe also contains these cell bodies (arrowhead) and axon bundle (large arrow). Scale bars=20 μm (C, D), 50 μm (F, H), and 100 μm (A, B, E, G).

in the nervous system of *S. litura* may be most active in late larval and adult stage. In other words, the actions of these neurons may become greatly limited in the nervous system of the pupae.

Kang and Lee (1997) reported patterns of postembryonic development of LK-LI neurons in the brain of *S. litura*. The LK-LI neurons which show larger numbers and stronger immunoreactivity in the larval brains were described to reduce in number and display weak immunoreactivity in both pupal and adult brains. The results obtained from the LK-LI neurons in the brains of larva and pupa are in part in accord with those from the LomTK I-IR neurons in the brains of larva and pupa. However, evidences from the LK-LI neurons which show both the reduction in number and weak immunoreactivity in the adult brain, are contrary to those obtained from the LomTK I-IR neurons in the adult brain, which display a rise in number and immunoreactivity.

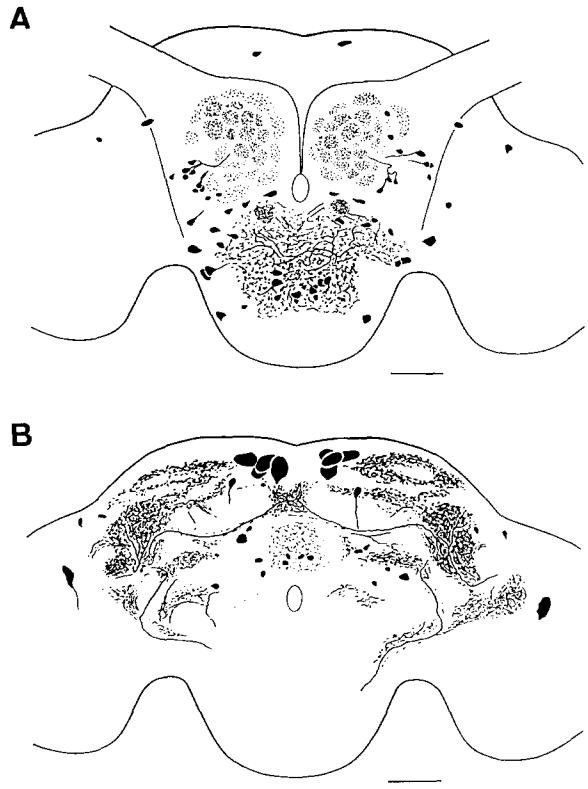

Fig. 7. Schematic representations for localization of these neurons in the adult brain. (A) Frontal view from whole brain. (B) Rear view from wholemount brain. Scale bars=100 μm.

The changes in number, structure, and localization of the LomTK I-IR neurons in the brains during the postembryonic development could not be easily interpreted in this investigation. According to a report by Cantera et al. (1994), however, during postembryonic development of corazonin-containing neurons and neurosecretory cells in *Phormia terraenovae*, the gradual aquisition of immunoreactivity by the LK-LI neurons in the brain can be interpreted as reflecting a differentiation progress of the LomTK I-IR neurons to an adult type. These neurons may be recruited from a larval subset of non-immunoreactive neurons, or they may be the cells from postembryonic proliferation. These suggestions must be supported in the future by further genetic and molecular biological investigations of both LomTK I-IR and LK-LI neurons in the postembryonic brains of insects.

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