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Apoptosis of neuronal cells in the brains of postembryonic silkworms *Bombyx mori* (Lepidoptera: Bombycidae)

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Key words. Silk moth, postembryonic life, brain, neuronal apoptosis, ecdysone, actinomycin, caspase

Abstract. The pattern and signal transduction of neuronal apoptosis in the brain of the silk moth, *Bombyx mori*, during postembryonic life, were characterized. Peak numbers of apoptotic neurons were detected in 4 day old 4th instar larvae, 9 day old 5th instar larvae and 4 day old pupae, indicating three waves of neuronal apoptosis during postembryonic development. Most of the apoptotic neurons were in the lateral portions of the brain. No apoptotic neurons were detected in 1 day old 1st instar larvae or in 7 day old pupae to 1 day old adults. Injection of 20-hydroxyecdysone (20E) into larvae resulted in a substantial increase in the brain in both neuronal apoptosis and cleavage of procaspases-8 and -3 into caspases-8 and -3. However, the injection of larvae with actinomycin D or cycloheximide inhibited death of pre-apoptotic neurons. Both the cleavage of procaspases-8 and -3 and death of pre-apoptotic neurons were inhibited by a general caspase inhibitor and caspase-8 and -3 inhibitors injected into larvae. These results suggest that 20E triggered the synthesis of a new protein that, in turn, induces cleavage of procaspases-8 and -3 into caspases-8 and -3. These caspases are prerequisites for neuronal apoptosis in postembryonic brains.

INTRODUCTION

Apoptosis, or programmed cell death (PCD), eliminates unwanted cells during normal development in animals (Jacobson et al., 1997; Jiang et al., 1997). Tissues in the larvae and pupae of holometabolous insects undergo massive degeneration. During the normal postembryonic development of these insects, apoptosis removes "pre-apoptotic" cells from a variety of tissues, including intersegmental muscles (Lockshin & Williams, 1965; Schwartz & Truman, 1982), central nervous system (CNS) (Robinow et al., 1997; Hoffman & Weeks, 1998; Draizen et al., 1999), prothoracic glands (Dai & Gilbert, 1997), midgut and salivary glands (Jiang et al., 1997; Lee et al., 2002), retina (Miller & Cagan, 1998; Yu et al., 2002) and silk gland (Chinzei, 1975; Terashima et al., 2000).

During postembryonic development, a number of neurons in the insect brain undergo apoptosis. In the adult brain of *Drosophila* (Truman et al., 1992, 1994) and pupal brain of *Apis mellifera carnica* (Ganeshina et al., 2000), some neurons undergo programmed death, suggesting a characteristic pattern with regard to the number of the pre-apoptotic neurons. To date, however, little is known about neuronal apoptosis in postembryonic brains of insects.

It is suggested that 20E triggers cell death in *Drosophila* and the midgut, salivary gland, silk gland and wings of silkworms (Terashima et al., 2000; Fujiwara & Ogai, 2001; Tsuzuki et al., 2001; Lee et al., 2002). However, it is unclear whether 20E functions as a critical exogenous stimulus for neuronal apoptosis in the brains of postembryonic insects.

Cysteine proteases of caspases perform an essential function in PCD in metazoans (Henkart, 1996; Enari et al., 1998; Mancini et al., 1998; Guo & Hay, 1999; Ferri & Kroemer, 2001; Wang, 2001). Among the wide range of metazoan cells that undergo apoptosis by activation of a few signal transduction pathways, some appear to induce their own death by activating both caspases-8 and -3 after receiving exogenous apoptotic cues (Mehmet, 2000). In *Drosophila* cells, caspases are expressed by genes such as drICE (Fraser & Evan, 1997) or dronc (Dorstyn et al., 1999), which also appear to participate in apoptotic signal transduction. In particular, the exposure of the second instar larval salivary glands and midgut to ecdysone results in a massive increase in dronc (a *Drosophila* caspase) mRNA levels, which eventually trigger cell death (Dorstyn et al., 1999). The *Drosophila* retinal neurons and accessory planta retractor (APR (6)) motoneurons in abdominal segment six of *Manduca sexta* undergo programmed death triggered by caspase or caspase-3 (Yu et al., 2002; Kinch et al., 2003). However, few studies have attempted to elucidate the process by which neuronal

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apoptosis is induced by caspases in insect brains during metamorphosis.

Microtubule-associated protein 2 (MAP2) (Izant & McIntosh, 1980; Binder et al., 1984; Caceres et al., 1984) and neuron-specific enolase (NSE) (Schmechel et al., 1978; Vullings et al., 1989) are used as molecular markers of neurons in the nervous system of mammals and insects. In particular, MAP2 is a specific marker of neurons and is found exclusively in differentiated neuronal cells (Izant & McIntosh, 1980). In immuno-labelled rat brain, MAP2 monoclonal antibodies stain only neurons, not the glial cells (Binder et al., 1984). Within neuronal cells, more MAP2 is present in dendrites and cell bodies than in axons (Caceres et al., 1984).

Apoptosis is associated with nuclear DNA fragmentation (Gavrieli et al., 1992; Oberhammer et al., 1993). Using the TUNEL assay, cells that undergo apoptosis by DNA fragmentation can be visualized at the single-cell level (Gavrieli et al., 1992). Within cells of tissues that undergo apoptosis, only nuclei located at positions where apoptosis is expected are found to be stained when examined under a confocal microscope.

Unlike most other animals, holometabolous insects, such as *B. mori*, undergo a metamorphosis, which is accompanied by dramatic changes in external and internal features during the transformation of larvae into adults. These changes include the neuronal apoptosis of brains during the transformation of larva to pupa (Truman et al., 1993). Since earlier studies on neuronal apoptosis concentrated on specific neurons of the *M. sexta* ventral ganglia in primary culture using 20E (Truman, 1983; Truman & Schwartz, 1984; Levine & Truman, 1985; Prugh et al., 1992; Weeks & Davidson, 1994; Streichert & Weeks, 1995; Sandstrom & Weeks, 1996; Streichert et al., 1997; Hoffman & Weeks, 1998; Kraft et al., 1998; Hoffman & Weeks, 2001; Zee & Weeks, 2001; Kinch et al., 2003), it is now important for insect neurobiologists to understand the pattern or mechanism of neuronal apoptosis in the brain during postembryonic development. The silkworm, *B. mori*, is recognized by entomologists as one of the most easily-reared and widely-used model insects. In particular, the pattern of neuronal apoptosis during the transformation from larval to pupal brains is poorly understood in insect species.

In this paper the pattern of neuronal programmed death in the postembryonic silkworm brain and induction of neuronal programmed death in the brain by 20E are described, together with the involvement of caspases-8 and -3 in the signal transduction pathway for neuronal programmed death.

MATERIAL AND METHODS

Animals

Cold-treated eggs of the silk moth, *Bombyx mori* (Lepidoptera: Bombycidae), were kindly supplied by the National Institute of Agricultural Science and Technology (Suweon, Korea). After incubation for about 10 days at 26.5°C the eggs hatched and the time and date of hatching were recorded on the plastic containers in which they were reared on the artificial diet "Silk-mate" (Nihon Nosan Kogyo, Yokohama, Japan) at 25 ± 1°C, a 12L : 12D photoperiod and 70% humidity in growth chambers (Doori, Seoul, Korea) (Mizoguchi et al., 2001).

The larvae passed through 5 instars and then metamorphosed into pupae, finally emerging as adults. The first instar larval stage lasted for 4 days, the 2nd 3 days, 3rd 4 days, 4th 4 days, 5th 9 days and pupae 9 days. The majority of 5th instar larvae began to wander on day 5 (first gate) or 6 (second gate) and pupated on either day 9 or 10, depending on the batch. First-gate and day-9 animals were used to monitor the pattern of neuronal apoptosis and for inducing or inhibiting it using 20E or other chemicals. For monitoring the pattern of neuronal apoptosis or tracing the results after in vivo injection of 20E and other chemicals, larvae, pupae and adults 12 h after hatching or a particular ecdysis were selected from the rearing stock.

Combined immunofluorescent MAP2 labelling and TUNEL assay

To trace the pattern of neuronal apoptosis throughout postembryonic life, animals were collected from the culture just after hatching or a particular ecdysis (Fig. 2A).

Prior to dissection, animals were kept at 4°C for about 1 h and all dissections were done in a cold room. Under a stereoscope, brains were isolated by careful removal of the outermost cuticle or lens from larvae, pupae and adults in 0.1 M sodium phosphate buffer (PB, pH 7.4). Isolated brains were fixed in 4% paraformaldehyde (PFA) in 0.1 M PB for 6 to 10 h at 4°C, depending on the quantity of tissue available for analysis (Kim et al., 2004; Park et al., 2004).

For combined immunofluorescent MAP2 labelling and subsequent TUNEL assay a slightly modified form of Ray et al. (2000) method was used. To reduce non-specific background, rehydrated brains were blocked with 200 µl of 2% rabbit preimmune serum in phosphate buffered saline (PBS) for 30 min at room temperature. This solution was then replaced with 200 µl PBS solution containing (1 : 200) mouse anti-MAP2 primary monoclonal antibody (Chemicon, CA, USA); the brains were then incubated for 1 day in a cold chamber, followed by two washes in PBS for 10 min each. The brains were then rinsed with 200 µl PBS solution that contained a mixture of Cy3-conjugated rabbit polyclonal anti-mouse IgG antibody (1 : 500; Sigma) for 1 day in a cold chamber in darkness with agitation at 100 rpm. The brains were washed twice in PBS for 10 min each. In order to visualize cells that underwent apoptosis, the TUNEL method was subsequently applied. The brains were digested in 20 µl of 20 µg/ml proteinase K solution in PBS for 10 min and then washed for 5 min with PBS at room temperature. Thereafter, the fixed brains were treated according to the TUNEL assay kit protocol (Promega, Madison, USA).

Horizontal 5 µm thick serial sections of brains treated with combined immunofluorescent MAP2 labelling and TUNEL assay were examined under a confocal microscope (Zeiss LSM 310). Multi-focused pictures of all the serial sections of the brains were saved in a computer and the number of apoptotic neurons in the sections of each of the brains were compared, and if necessary by using printed pictures of the brain sections. Statistical analysis of the numbers of apoptotic neurons in the brains was performed using the SPSS 12.0 program.

Immunogold electron microscopy

Brains were dissected from 3 day old 4th instar larvae and fixed for 1 h at 4°C in 0.1 M PB (pH 7.4) containing 4% PFA. Fixed brains were washed with the same buffer and post-fixed for 1 h in the same buffer containing 1% OsO_4. Subsequently, tissues were dehydrated using a graded series of alcohol and then acetone, and embedded in Epon-Araldite. Semi-thin and

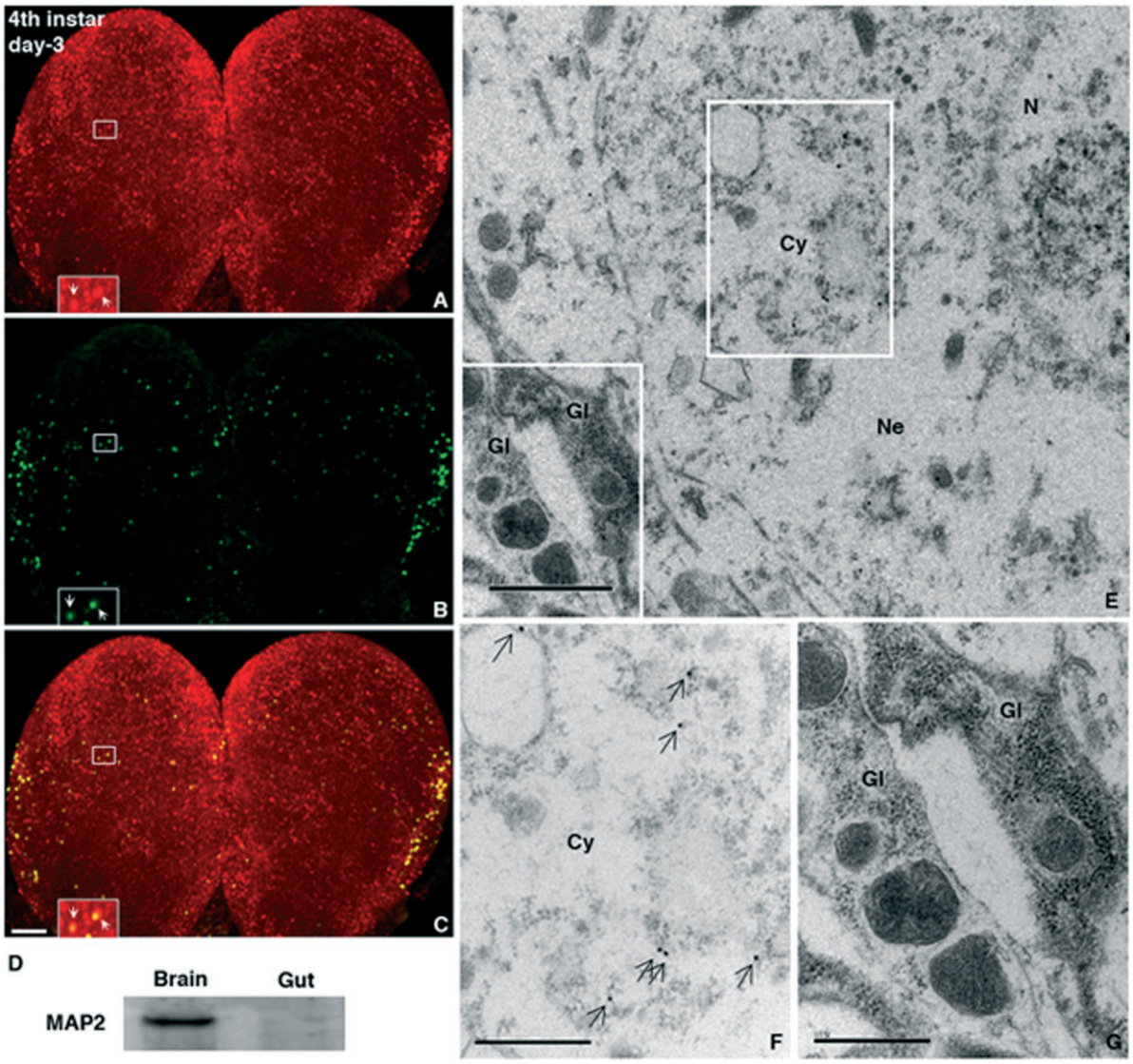

Fig. 1. The combined use of immunofluorescent MAP2 labelling and TUNEL assay (modified after Ray et al., 2000), with anti-MAP2 conjugated with Cy3 as a neuronal marker (A–C, each of the three photographs represent one of a series of mutli-focused sections), Western blotting (D) and immunogold staining (E–G) to demonstrate that all the 130 apoptotic cells in the brain of a 3 day old 4th instar larva of *B. mori* are neurons. All normal cells in the brain appear red when stained with anti-MAP2 (A), whereas the nuclei of apoptotic cells appear as light green spots after staining with FITC. Apoptotic neurons have red cytoplasm and yellow nuclei when MAP2 is merged with FITC. See the two stained cells (arrows) in the three photographs on the left of the figure. Scale bar on Fig. 1C is 60 µm and indicates the magnification of the two photographs in Fig. 1A and 1B. (D) The Western blotting indicates that the brain of 3 day old 4th instar larvae but not the mid-gut of 3 day old 4th instar larvae contained MAP2. (E) MAP-2-like immunogold staining of a neuron (Ne) and glial cells (Gl) in the middle lateral portion of the brain of a 3 day old 4th instar larva. The two insets in this photograph are magnified in the next two photographs (F and G). Cy, cytoplasm of neuron; N, nucleus. Scale bar indicates 1 µm. (F) There are many gold particles (thin arrows) in the cytoplasm (Cy) of this cell. Scale bar is 0.5 µm. (G) Glial cells (Gl) adjacent to neurons do not contain gold particles. Scale bar is 0.5 µm.

ultra-thin sections were prepared from a middle lateral portion of the brain using an ultramicrotome (RMC MT-X). Semi-thin tissue sections were stained with 1% toluidine blue-borax solution. Ultra-thin sections were mounted on copper grids and blocked with the same buffer containing 2% BSA and 1% Tween-20. Tissues on grids were stained with anti-MAP2 primary antibody (monoclonal, Chemicon), then labelled with gold-conjugated secondary antibody (BBInternational UK) and finally stained with uranyl acetate. Tissues on grids were examined under an electron microscope (Philip T12). This immunogold staining procedure was modified after Dai et al. (1995).

Isolation of brain and midgut for MAP2 detection and Western blotting

Three day old 4th instar larvae were dissected under a stereoscope, and the brain and midgut were isolated in 0.1 M PB (pH 7.4). Isolated tissues were washed in fresh PB, homogenized in lysis buffer (Narkilahti et al., 2003) and then centrifuged. Protein samples were separated on 12% SDS-PAGE and then transferred to nitrocellulose membranes. The rest of the procedure followed the method described by Yakovlev et al. (2001).

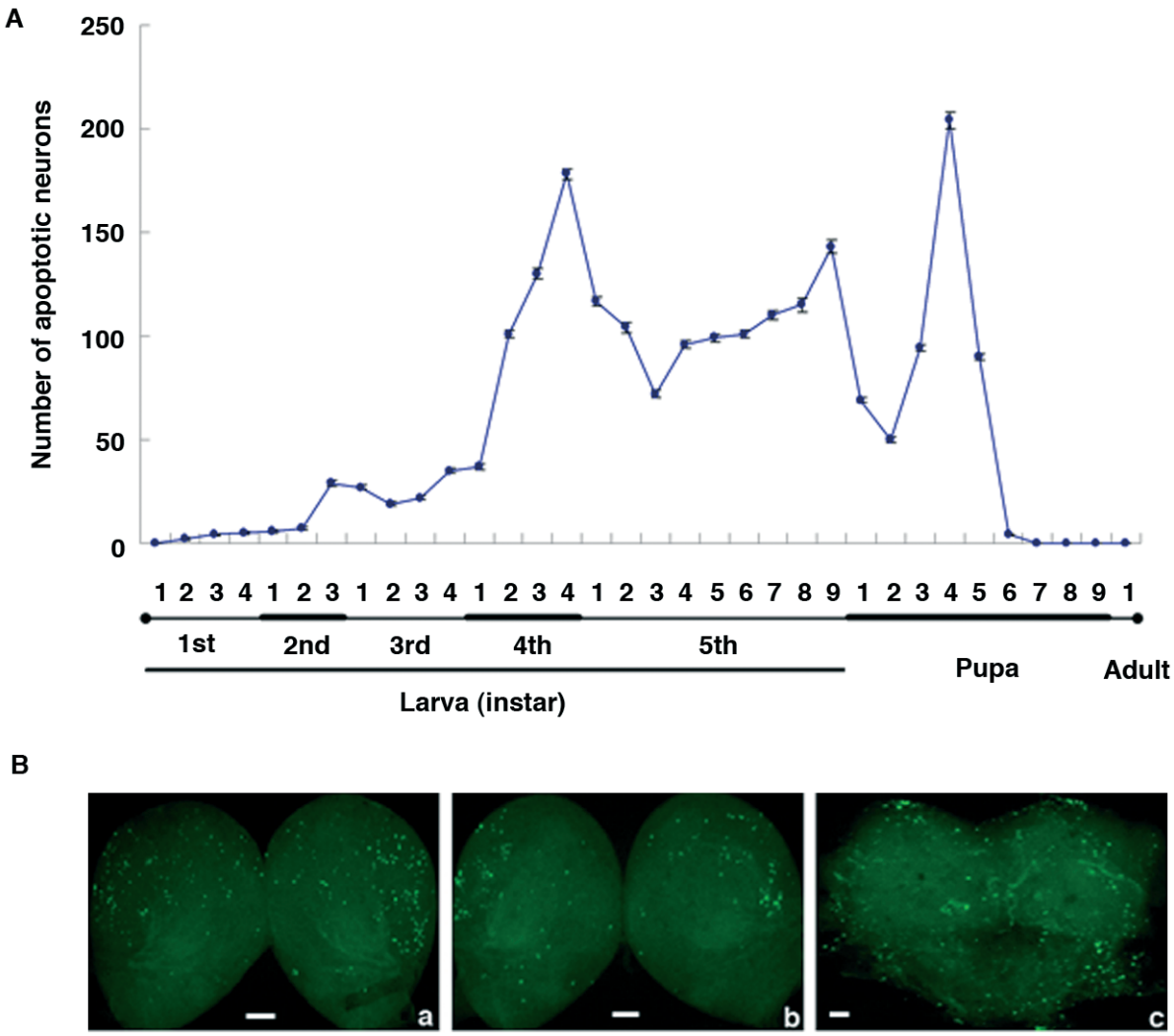

Fig. 2. The trend in time of the number of apoptotic neurons in the brains of postembryonic silkworms and photographs of the peak levels of apoptotic neurons recorded at three stages during development. (A) Trend in the number of apoptotic neurons in brains of postembryonic silkworms (all $p < 0.01$). The largest numbers of apoptotic neurons were recorded in 4 day old 4th instar larvae, 9 day old 5th instar larvae and 4 day old pupae. (B) Brains of a 4 day old 4th instar larva, a 9 day old 5th instar larva and a 4 day old pupa showing peak levels of apoptotic neurons. Viewed under under a confocal microscope all brains stained using FITC of combined immunofluorescent MAP2 labelling and TUNEL assay, appear green and the nuclei of apoptotic neurons as light green spots. Four day old 4th instar (a) and 9 day old 5th instar (b) larvae and 4 day old pupae (c) had approximately 178, 143 and 204 apoptotic neurons in their brains, respectively (see Fig. 2A for more detailed data). Apoptotic neurons were mainly located in the middle and lateral regions of the brains of 4 day old 4th instar larvae, upper and middle lateral regions in 9 day old 5th instar larvae, and medial, upper and lower lateral regions (suboesophageal ganglion) in 4 day old pupae. Scale bars are 60 µm.

Injection of 20E and other chemicals

The 20E was dissolved in ethanol at a concentration of 10 mg/ml and stored at –20°C until required. To determine the amount of 20E (Sigma) required to induce neuronal apoptosis in the brains of larvae, 0.1, 1, 5 or 10 µg/ml of 20E, prepared from a stock solution, were injected through the body wall into the hemolymph near the brain, per gram of body weight of 2 day old 3rd instar larvae (with an average body weight of 0.1 g; physiological level of 20E is unknown), 2 day old 4th instar larvae (average of 0.5 g; physiological level of 20E is about 0.1 µg/ml) and 1 day old 5th instar larvae (average of 1 g; physiological level of 20E is close to 0 µg/ml) (Mizoguchi et al., 2001), using a Hamilton syringe (Truman, 1993). The same volume (1 µl) of each 20E solution was injected in each animal.

Forty-eight hours after injecting 20E the brain was carefully isolated from the larvae in the three stages of development specified above and the apoptotic neurons labelled by combined immunofluorescent MAP2 labelling and subsequent TUNEL assay.

To clarify whether inhibition of RNA or protein synthesis affected apoptosis of neurons in brains of postembryonic individuals they were injected with actinomycin D (ActD, Sigma) or cycloheximide (CHX, Sigma, Missouri, USA). These substances were dissolved in ethanol at concentrations of 0.1 or 10 mg/ml, respectively (Hoffman & Weeks, 2001), and stored at –20°C. A 1 µl volume containing 100 ng ActD or 10 µg CHX, derived from stock solutions, was injected into the hemolymph of 2 day old 4th instar larvae. Twenty-four hours later the brains were carefully isolated from the larvae and subjected to the Western blotting procedure, as described below, or the brains were carefully isolated from the larvae 48 h later and subjected

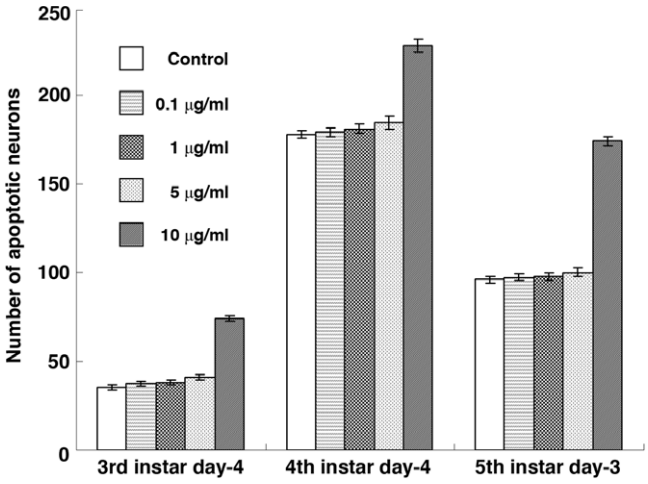

Fig. 3. Number of apoptotic neurons developing in the brains of larvae injected with 20E. Histogram shows the numbers of apoptotic neurons in brains 48 h after injecting 1 μl each of either 0.0, 0.1, 1, 5 or 10 μg/ml of 20E into 2 day old 3rd instar larvae, 2 day old 4th instar larvae or 1 day old 5th instar larvae. Neuronal apoptosis was induced by injections of 10 μg/ml but not 0.0, 0.1, 1 or 5 μg/ml. In order to induce neuronal apoptosis, therefore, 10 μg/ml of 20E were injected. Compared to the brains of control larvae those of larvae injected with 20E had a greater number of apoptotic neurons, 111%, in 4 day old 3rd instar larvae ($p < 0.01$), and fewer in 4 day old 4th instar larvae (28% , $p < 0.05$) and 3 day old 5th instar larvae (81%, $p < 0.05$). Number of samples in each test was ten.

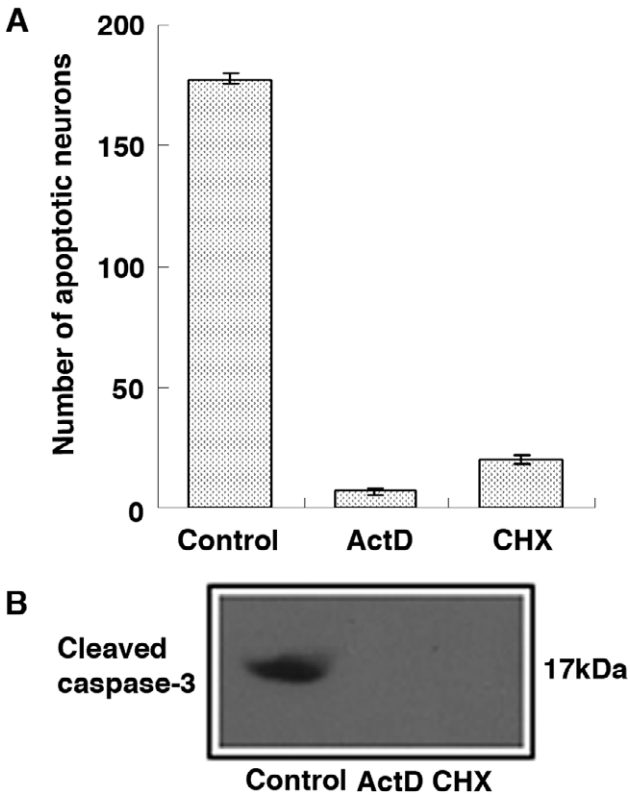

Fig. 4. Actinomycin D (ActD) and cycloheximide (CHX) block apoptosis of pre-apoptotic neurons in the brains of silkworm either by inhibiting RNA transcription or the synthesis of protein. A – Histogram showing the numbers of apoptotic neurons present in brains 48 h after injecting either ActD or CHX. The brains of injected larvae had approximately 7 (a 96% decrease when injected with ActD) or 20 (an 89% decrease when injected with CHX) apoptotic neurons compared with the 178 in the brains of the control group (both $p < 0.05$). B – Western blots of the brains of 2 day old 4th instar larvae 24 h after injection with 1 μl per individual of 100 ng ActD or 10 μg CHX. Results suggest that cleavage of procaspase-3 into caspase-3 was completely inhibited by both these chemicals. For each the number of samples is ten.

to combined immunofluorescent MAP2 labelling and subsequent TUNEL assay.

In order to determine whether a general caspase, caspase-8, or caspase-3 was involved in neuronal apoptosis in the brains of postembryonic individuals, larvae were injected with caspase inhibitors. The caspase inhibitor (Z-Asp-CH_2-DCB, Calbiochem), caspase-8 inhibitor (Z-IETD-FMK, Calbiochem) and caspase-3 inhibitor (Ac-DEVD-CMK, Calbiochem) were dissolved in dimethyl sulfoxide at a 100 mM concentration and stored at –20°C until used (Terashima et al., 2000). A dose of 25 μM of general caspase inhibitor, 50 μM of caspase-8 inhibitor or 25 μM of caspase-3 inhibitor, which were prepared from the stock solutions, were injected into the hemolymph of 2 day old 4th instar larvae, each in a volume of 1 μl.

Forty-eight hours after injection of the general caspase inhibitor, caspase-8 inhibitor, or caspase-3 inhibitor, the brains were carefully isolated from the larvae and subjected to combined immunofluorescent MAP2 labelling and subsequent TUNEL assay. For Western blotting, the brains were isolated from the larvae 24 h after injecting the caspase inhibitors.

To determine when after injection caspase-3 (Cell Signaling) was cleaved from procaspase 3 (Santa Cruz Biotechnology), 10 μg/ml 20E were injected into the hemolymph of 2 day old 4th instar larvae, and their brains carefully isolated 0, 24 or 36 h after the 20E injection. The isolated brains were then subjected to the Western blotting procedure. For the control, PBS, instead of 20E, was injected into 2 day old 4th instar larvae.

RESULTS

Demonstration of apoptotic neurons

The fact that all apoptotic cells found in the brain are neurons was confirmed by combined immunofluorescent MAP2 labelling and subsequent TUNEL assay, Western blotting and immunogold electron microscopy.

After the combined immunofluorescent MAP2 labelling and subsequent TUNEL assay of the brain of 3 day old 4th instar larvae the nuclei and cytoplasm of all the neuronal cells located in the cortex of the two cerebral halves were coloured red (Fig. 1A, two magnified stained cells from the left half of the brain). The nuclei of all the apoptotic cells were stained green by FITC (Fig. 1B, two magnified stained cells from left half of brain). When the MAP2 and FITC stained images were superimposed, the nuclei of the apoptotic neurons appeared as yellow spots in the centers of the cells and the cytoplasm was stained red (Fig. 1C, two magnified stained cells from the left half of the brain). In the brain of a 3 day old 4th instar larva there were about 130 yellow spots and neighbouring red structures.

The analyses of Western blots revealed that the brain of 3 day old 4th instar larvae was MAP2 positive but not the

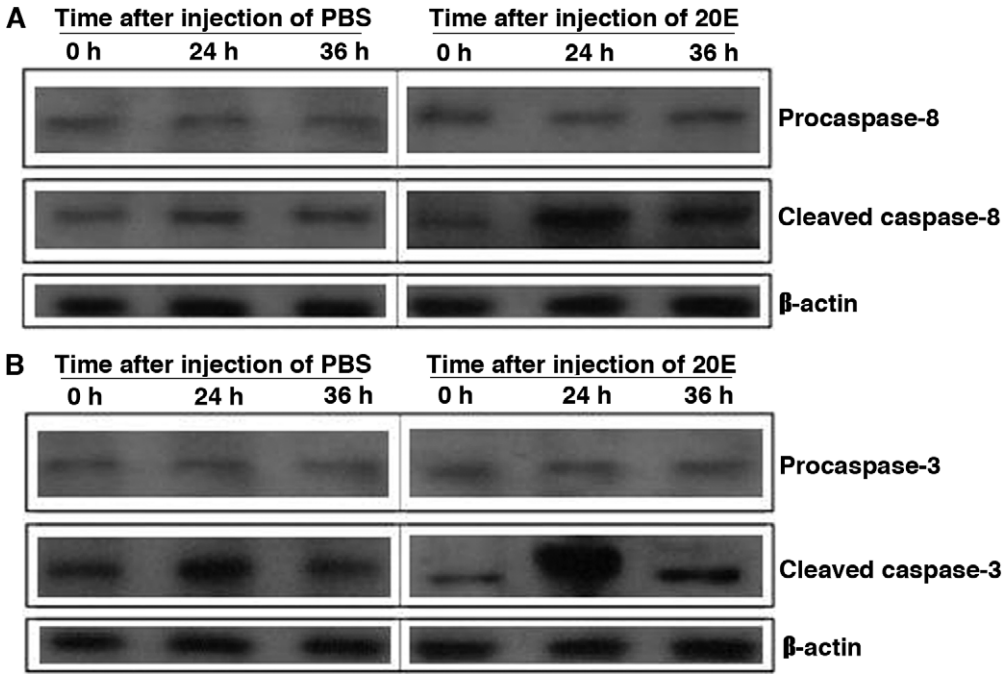

Fig. 5. Western blotting used to detect cleavage of procaspases-8 and -3 into caspases-8 and -3 in the brains of 2 day old 4th instar larvae previously injected with 10 µg/ml of 20E. A – Western blots of brains of larvae showing the presence of cleaved caspase-8 at 0, 24 and 36 h after injection with 20E. The quantity of procaspase-8 did not change but that of cleaved caspase-8 peaked 24 h after the injection and was still high after 36 h. B – Western blots of the brains of larvae showing presence of cleaved caspase-3 at 0, 24 and 36 h after injection with 20E. There was no change in the level of procaspase-3 following the injection of 20E. In contrast, the cleavage of procaspase-3 into an active fragment peaked 24 h after injection.

gut (Fig. 1D). The immunogold electron microscopy of the brain of 3 day old 4th instar larvae revealed that gold particles were only associated with anti-MAP2 in neurons. In Fig. 1E the gold particles are present in the cytoplasm of normal and apoptotic neurons but not the nuclei (Fig. 1F). Gold particles were not found in the cytoplasm or nucleus of the glial cells (Fig. 1G). These results suggest that MAP2 protein was located in the cytoplasm of the neurons in the brains of 3 day old 4th instar larvae, but not in the glial cells. Therefore, all cells in the brains of postembryonic individuals that had yellow coloured nuclei and red coloured cytoplasm, following combined immunofluorescent MAP2 labelling and the TUNEL assay, can be described as neurons.

Pattern of apoptotic neurons in postembryonic brains

Horizontal serial sections, each 5 µm thick, through the brains of postembryonic individuals, treated by combined immunofluorescent MAP2 labelling and TUNEL assay were examined from top to bottom under a confocal microscope. Features of apoptotic neurons in each brain, along with those of normal neurons, were saved as serial photographs in a computer. The number of apoptotic neurons in each brain was counted by comparing photographs of the serial sections using a computer. If necessary, the counting of apoptotic neurons was done on hard copies of the photographs. Therefore, the number of apoptotic neurons in each brain was either obtained from the photographs of the serial sections stored on a computer or from printed photographs.

In the brain of 1 day old 1st instar larvae there were no apoptotic neurons, but a few are present in the brains of 2 day old 1st instar larvae. There was a gradual but small increase in the number of apoptotic neurons between the 2nd day of the lst instar and the 2nd day of 2nd instar. However, there was a substantial increase in the number of apoptotic neurons to approximately 29 and 35, respectively, in the brains of 3 day old 2nd instar larvae and 4 day old 3rd instar larvae (Fig. 2A).

There were substantial numbers of apoptotic neurons in the brains of 4 day old 4th instar and 9 day old 5th instar larvae, especially in the lateral portions of their brains. There was a remarkable decrease in the number of apoptotic neurons in the brains of pupae. In 4 day old pupae there were approximately 204 apoptotic neurons but none were detected in the brains of 7 day old pupae and 1 day old adults. As shown in Fig. 2A three peaks in the numbers of apoptotic neurons were detected, the first in the brains of 4 day old 4th instar larvae, the second in 9 day old 5th instar larvae and third in 4 day old pupae.

The average number of apoptotic neurons present during post-embryonic development shown in Fig. 2A was determined by dividing the total number of apoptotic neurons recorded by the number of brains examined at each stage. The difference between the least and greatest number of apoptotic neurons recorded for the same postembryonic stage ranged from 1 to 5. The pattern of apoptosis in postembryonic life was based on examining 599 brains. Most of the apoptotic neurons were recorded in the larval stages with a few in the pupal stage.

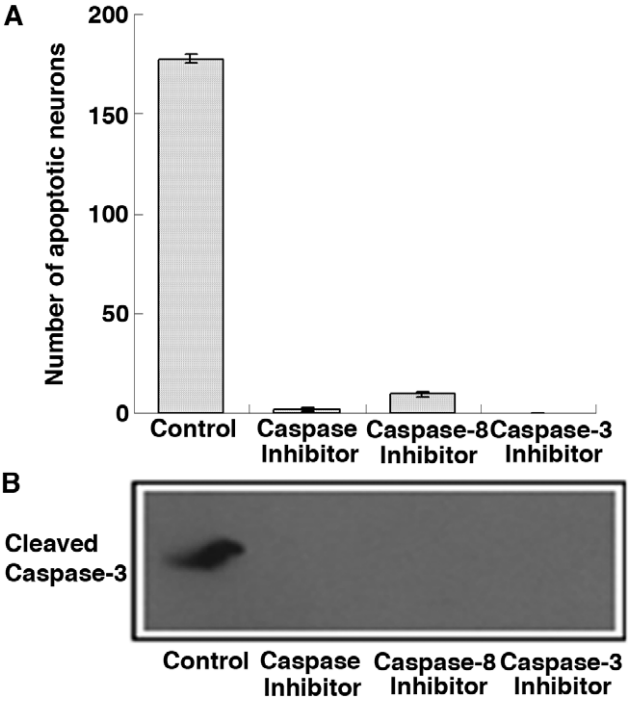

Fig. 6. Neuronal apoptosis in the brains of *B. mori* inhibited by injection of inhibitors of general caspase, and caspases-8 and -3. A) The number of apoptotic neurons in the brains 48 h after injection ($p < 0.05$). B) Western blots of the brains of 2 day old 4th instar larvae showing presence of cleaved procaspase-3 24 h after injecting the larvae with 25 μM of general caspase inhibitor, 50 μM caspase-8 inhibitor, or 25 μM caspase-3 inhibitor compared with that in the control. The volume of each inhibitor injected was 1 μl per individual. For each the number of samples is ten.

Apoptotic neurons were evenly distributed throughout the brains of 3 day old 2nd instar and 4 day old 3rd instar larvae (data not shown), but they were mainly distributed in the middle and lateral regions of the brains of 4 day old 4th instar larvae, upper and middle lateral regions of the brains of 9 day old 5th instar larvae, and medial, upper and lower lateral portions of the brains (suboesophageal ganglion) of 4 day old pupae (Fig. 2B).

Induction of neuronal apoptosis in larval brains by injection of 20E

No changes were noted in the hemolymph of 2 day old 3rd instar larvae, but an increase in the titer of 20E is recorded in 2 day old 4th instar larvae and a decrease in 1 day old 5th instar larvae (Mizoguchi et al., 2001). There were no apparent changes in the numbers of apoptotic neurons in larvae injected with 0.1, 1 or 5 μg/ml 20E, compared to the controls. However, the number of apoptotic neurons increased significantly in the brains of larvae after injection of 10 μg/ml 20E (Fig. 3).

When 0.1, 1 and 5 μg/ml 20E were injected into 2 day old 3rd instar larvae the number of apoptotic neurons was comparable (about 37 with 0.1 μg/ml, a 5.7% increase) or slightly different (about 38 with 1 μg/ml, a 8.6% increase; about 41 at 5 μg/ml, a 17.1% increase) from the number in the control animals (about 35). When larvae were injected with 10 μg/ml 20E, however, the number of apoptotic neurons increased significantly (about 74, 111.4% increase).

When 0.1, 1, 5 and 10 μg/ml of 20E were injected into 2 day old 4th instar larvae and 1 day old 5th instar larvae, similar dose-dependent results to those described above were recorded (see Fig. 3).

Our results strongly suggest that most of the neuronal apoptosis in the brains of larvae is triggered by increases in the titer of 20E in the hemolymph and this might also be true for the brains of pupae.

Effects of ActD and CHX on neuronal apoptosis in the brains of larvae

The number of apoptotic neurons in the brains of larvae injected with ActD was significantly lower (about 7 neurons and 96.1% decrease) than in the control (about 178 neurons) (Fig. 4A). When animals were injected with CHX the number of apoptotic neurons decreased significantly (about 20 neurons and 88.8%) compared to the control (about 178 neurons). Western blotting of brain lysates, performed 24 h after ActD or CHX injection, indicated no caspase-3 was cleaved in the brains of injected animals (Fig. 4B). These results imply that specific RNA or protein synthesis in the pre-apoptotic brain neurons, induced by 20E, might be the cause of neuronal death.

20E injection induced the cleavage of procaspase-8 and -3 into active caspases

The brain of larvae injected with 20E consistently contained unchanged levels of procaspase-8 (Fig. 5). In contrast, the cleavage of procaspase-8 into caspase-8 occurred at high levels 24 h after injection (Fig. 5A). Caspase-8 was still present after 36 h.

High levels of caspase-3 were detected in the brains of larvae 24 h after they were injected with 10 μg/ml 20E (Fig. 5B) but the level had decreased by 36 h. In contrast, procaspase-3 levels were consistently unchanged following injection of 20E.

Procaspases-8 and -3 levels in the brains of larvae injected with 20E were very similar to those in the brains of larvae injected with PBS. However, levels of cleaved caspase-8 and -3 in the brains of larvae injected with 20E were higher at 24 and 36 h than in the brains of larvae injected with PBS.

Effects of inhibitors of general caspase, caspase-8 and caspase-3 on neuronal apoptosis in the brains of larvae

The number of apoptotic neurons in the brains of 2 day old 4th instar larvae was significantly reduced 48 h after injection with 25 μM Z-Asp-CH_2-DCB, 50 μM Z-IETD-FMK or 25 μM Ac-DEVD-CMK. However, the number of apoptotic neurons did not increase in the brains of sham-injected larvae (control). When larvae were injected with the caspase inhibitors, the number of apoptotic neurons decreased significantly to about 2 (98.9% decrease), 9 (94.9%) or 0 (100%), respectively (about 178 in control) (Fig. 6A). Western blotting of

lysates of brains 24 h after injection of the three inhibitors yielded no evidence of caspase-3 (Fig. 6B).

DISCUSSION

Based on these results a hypothetical model of a signaling pathway for neuronal apoptosis initiated by increases in the titer of 20E in the brains of post-embryonic individuals of *B. mori* is proposed. After binding to a receptor in the cytoplasm of pre-apoptotic neurons, 20E is thought to enter the nuclei and stimulate the expression of a specific gene. A newly synthesized protein is thought to convert inactive procaspase-8 into active caspase-8, which transforms procaspase-3 into cleaved caspase-3. This enzyme may also be involved in subsequent reactions. Caspase-3 activation may result in DNA fragmentation and eventual induction of the death of neurons.

Demonstration of apoptotic neurons

MAP2 or NSE has been used to stain neurons in mammals (Schmechel et al., 1978; Izant & McIntosh, 1980; Binder et al., 1984; Caceres et al., 1984) and insects (Vullings et al., 1989). In brain tissue that contains significant amounts of MAP2, only differentiated neurons, not glial cells, contain MAP2 (Izant & McIntosh, 1980). MAP2 monoclonal antibodies stain neurons but not glial cells in rat brains (Binder et al., 1984). In neurons, MAP2 interacts with microtubules, neurofilaments and actin filaments. As shown in Fig. 1, MAP2 was only present in the cytoplasm of the neurons in the brain.

The TUNEL assays revealed that apoptosis is associated with the fragmentation of nuclear DNA in neurons in the brains of silkworms. This method revealed the individual neurons that underwent PCD by DNA fragmentation. Only the nuclei of neurons undergoing apoptosis were yellow when the MAP2 and FITC stained images were superimposed under a confocal microscope.

Pattern of apoptotic neurons in the brains of postembryonic individuals

Many neurons in the brain underwent apoptosis during most metamorphic processes. In particular, a large number of apoptotic neurons were detected in the brains of 4 day old 4th instar and 9 day old 5th instar larvae (or 5 day old prepupae), and 4 day old pupae. These three peaks in the number of apoptotic neurons coincide with particular metamorphic stages in early development. However, there were no apoptotic neurons in the brains of 1 day old 1st instar larvae, late pupae, and 1 day old adults (see Fig. 2A).

These results suggest that during the normal development of the brain unnecessary neurons were removed by apoptosis. During development PCD is routinely used to delete unwanted structures, control cell numbers and eliminate harmful, abnormal, or mislocated cells (Jacobson et al., 1997; Miller & Cagan, 1998; Baehrecke, 2000; Ganeshina et al., 2000; Martin & Baehrecke, 2004). In the postembryonic brains of *B. mori*, apoptosis served to delete redundant neurons and sculpture newly needed neurons.

Induction of neuronal apoptosis in the brains of larvae injected with 20E

During the metamorphic reorganization of an insect's CNS, fluctuations in the titer of 20E in the hemolymph tend to evoke a broad spectrum of cellular responses, including neuronal apoptosis, cell proliferation, maturation, and remodelling of larval neurons into adult neurons (Schubiger et al., 1998).

In *B. mori*, the secretion of 20E by the prothoracic gland results in three peaks in the concentration of this substance in the hemolymph between the 4th instar and 1 day old adult: the first, the smallest peak occurred between the 2nd and 3rd day of the 4th instar, the second, an intermediate sized peak occurred between the 7th and 8th day of the 5th instar, and the largest peak occurred between the 2nd and 3rd day of the pupal stage (Mizoguchi et al., 2001).

The three peaks in the numbers of apoptotic neurons in the brains each occurred approximately 1.5 days after the three peaks in the hemolymph titers of 20E (Fig. 2). As the titers of 20E recorded in the hemolymph in our study are similar it is likely that this substance regulates neuronal apoptosis in the brain during post-embryonic development. There is evidence that changes in the titer of 20E induce apoptosis in other tissues of insects (Robinow et al., 1997; Dorstyn et al., 1999; Draizen et al., 1999; Terashima et al., 2000; Fujiwara & Ogai, 2001; Lee et al., 2002; Kinch et al., 2003).

Effects of ActD and CHX on 20E-induced neuronal apoptosis in the brains of larvae

Some pre-apoptotic cells undergo apoptosis without synthesizing new proteins and therefore they must be a constitutive part of the cell, or the death machinery, required for the induction of apoptosis (Weil et al., 1996). In these cells, proteins necessary for apoptosis are constitutively present, albeit in an inactive form. However, apoptosis that occurs during development or metamorphosis may require synthesis of new proteins (Burek & Oppenheim, 1996).

Injection of larvae with Act or CHX inhibited apoptosis in their brains (Fig. 3), indicating that, despite the effects of 20E on pre-apoptotic neurons, ActD and CHX can inhibit protein synthesis and block neuronal apoptosis. Apoptosis of pre-apoptotic neurons in the brain might be induced by the synthesis of new proteins initiated by exposure to 20E. This has been demonstrated in the accessory planta retractor (APR) motor neurons in 6th abdominal segment [APR(6)] of *M. sexta*, which undergo segment-specific apoptosis 24 to 48 h after pupal ecdysis in response to changes in the titer of 20E in the hemolymph (Hoffman & Weeks, 2001).

However, injections of CHX at the prepupal stage blocked the apoptosis of pre-apoptotic APR (6) motorneurons. No caspase-3 was detected by Western blot analysis after injecting 2 day old 4th instar larvae with either ActD or CHX,, which might indicate that both ActD and CHX can block the cleavage of procaspase-3

into caspase-3 and thereby prevent the apoptosis of neuronal cells.

Cleavage of procaspase-8 and -3 in the brains of larvae injected with 20E

Expression of procaspase-3 and its subsequent cleavage into caspase-3, which is the end reaction in the transduction of apoptosis (Mehmet, 2000), is initiated by particular signals: increase in the titer of 20E in *B. mori*, status epilepticus in rats (Narkilahti et al., 2003) or alcohol (Young et al., 2003) in mice. In the brains of larvae, cleavage of procaspases-8 and -3 into caspases-8 and -3 was triggered 24 h after the administration of 20E (Fig. 5A, B).

In the rat, caspase-3 protein expressed in the right mediodorsal nucleus of the thalamus results in a subsequent kainite-induced epilepticus, which peaks 16–24 h later. The cleavage of procaspase-3 into active caspase-3, which is triggered in the corresponding neurons signaled by SE, occurs 2 to 7 days after SE (Narkilahti et al., 2003). Administration of alcohol triggers widespread neuronal apoptosis in the brains of infant mice and rats 6–16 h following administration and this is accompanied by activation of caspase-3 (Young et al., 2003).

Effects of inhibitors of general caspase, caspase-8, and caspase-3 on the apoptosis of neurons in the brains of larvae

Confocal microscopy was carried out on the brains of larvae 48 h after injection of each of three inhibitors (Fig. 6). Very few pre-apoptotic neurons were detected after injecting each of the caspase inhibitors (Fig. 6A) and no caspase-3 activity was detected (Fig. 6B). These results indicate that both caspases-8 and -3 are involved in signalling 20E-induced apoptosis of neurons in the brain.

Signaling pathway of apoptosis of neurons in the brains of postembryonic individuals of *B. morii* followed an increase in the titer of 20E in the hemolymph. 20E appeared to bind to its receptor (EcR) in the cytosol of pre-apoptotic neurons, resulting in the formation of a 20E-EcR complex, which then enters the nuclei and binds to a gene that then express a specific protein. This newly synthesized protein converts procaspase-8 into active caspase-8, which transforms procaspase-3 into active caspase-3 (Mehmet, 2000; Kinch et al., 2003). The cleavage of procaspase-3 into caspase-3 triggers the reactions leading to the apoptosis of neurons. These results further our understanding of the apoptosis of neurons in the brains of insects.

In conclusion, these results define and increase our understanding of the pattern and mechanisms of the neuronal apoptosis occurring in the brains of postembryonic silkworms.

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REFERENCES

BAEHRECKE E.H. 2000: Steroid regulation of programmed cell death during Drosophila development. *Cell Death Differ*. **7**: 1057–1062.

BINDER L.I., FRANKFURTER A., KIM H., CACERES A., PAYNE M.R. & REBHUN L.I. 1984: Heterogeneity of microtubule-associated protein 2 during rat brain development. *Proc. Natl. Acad. Sci. U.S.A.* **81**: 5613–5617.

BUREK M.J. & OPPENHEIM R.W. 1996: Programmed cell death in the developing nervous system. *Brain Pathol*. **6**: 427–446.

CACERES A., BINDER L.I., PAYNE M.R., BENDER P., REBHUN L. & STEWARD O. 1984: Differential subcellular localization of tubulin and the microtubule-associated protein MAP2 in brain tissue as revealed by immunocytochemistry with monoclonal hybridoma antibodies. *J. Neurosci*. **4**: 394–410.

CHINZEI Y. 1975: Biochemical evidence of DNA transport from the silk gland to the fat body of the silkworm, Bombyx mori. *J. Insect Physiol.* **21**: 163–171.

DAI J.D. & GILBERT L.I. 1997: Programmed cell death of the prothoracic glands of Manduca sexta during pupal-adult metamorphosis. *Insect Biochem. Mol. Biol.* **27**: 69–78.

DAI J.L., MIZOGUCHI A., SATAKE S., ISHIZAKI H. & GILBERT L.I. 1995: Developmental changes in the prothoracicotropic hormone content of the Bombyx mori brain-retrocerebral complex and hemolymph: analysis by immunogold electron microscopy, quantitative image analysis, and time-resolved fluoroimmunoassay. *Dev. Biol*. **171**: 212–223.

DORSTYN L., COLUSSI P.A., QUINN L.M., RICHARDSON H. & KUMAR S. 1999: DRONC, an ecdysone-inducible Drosophila caspase. *Proc. Natl. Acad. Sci. U.S.A*. **96**: 4307–4312.

DRAIZEN T.A., EWER J. & ROBINOW S. 1999: Genetic and hormonal regulation of the death of peptidergic neurons in the Drosophila central nervous system. *J. Neurosci.* **38**: 455–465.

ENARI M., SAKAHIRA H., YOKOYAMA H., OKAWA K., IWAMATSU A. & NAGATA S. 1998: A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391**: 43–50.

FERRI K.F. & KROEMER G. 2001: Organelle-specific initiation of cell death pathways. *Nat. Cell Biol.* **3**: E255–263.

FRASER A.G. & EVAN G.I. 1997: Identification of a Drosophila melanogaster ICE/CED-3-related protease, drICE. *EMBO J.* **16**: 2805–2813.

FUJIWARA H. & OGAI S. 2001: Ecdysteroid-induced programmed cell death and cell proliferation during pupal wing development of the silkworm, Bombyx mori. *Dev. Genes Evol*. **211**: 118–123.

GANESHINA O., SCHAFER S. & MALUN D. 2000: Proliferation and programmed cell death of neuronal precursors in the mushroom bodies of the honeybee. *J. Comp. Neurol*. **417**: 349–365.

GAVRIELI Y., SHERMAN Y. & BEN-SASSON S.A. 1992: Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol*. **119**: 493–501.

GUO M. & HAY B.A. 1999: Cell proliferation and apoptosis. *Curr. Opin. Cell Biol.* **11**: 745–752.

HENKART P.A. 1996: ICE family proteases: mediators of all apoptotic cell death? *Immunity* **4**: 195–201.

HOFFMAN K.L. & WEEKS J.C. 1998: Programmed cell death of an identified motoneuron in vitro: temporal requirements for steroid exposure and protein synthesis. *J. Neurobiol*. **35**: 300–322.

HOFFMAN K.L. & WEEKS J.C. 2001: Role of caspases and mitochondria in the steroid-induced programmed cell death of a motoneuron during metamorphosis. *Dev. Biol*. **229**: 517–536.

IZANT J.G. & MCINTOSH J.R. 1980: Microtubule-associated proteins: a monoclonal antibody to MAP2 binds to differentiated neurons. *Proc. Natl. Acad. Sci. U. S. A.* **77**: 4741–4745.

JACOBSON M.D. WEIL M. & RAFF M.C. 1997: Programmed cell death in animal development. *Cell* **88**: 347–354.

JIANG C., BAEHRECKE E.H. & THUMMEL C.S. 1997: Steroid regulated programmed cell death during Drosophila metamorphosis. *Development* **124**: 4673–4683.

KIM J.H., PARK C.W., SONG H.Y., KIM M.Y., CHOI C.O. & LEE B.H. 2004: Effects of brain-derived neurotrophic factor on neurite growth of deutocerebral neurons in brain of the silk moth Bombyx mori. *Entomol. Res*. **34**: 113–122.

KINCH G., HOFFMAN K.L., RODRIGUES E.M., ZEE M.C. & WEEKS J.C. 2003: Steroid-triggered programmed cell death of a motoneuron is autophagic and involves structural changes in mitochondria. *J. Comp. Neurol*. **457**: 384–403.

KRAFT R., LEVINE R.B. & RESTIFO L.L. 1998: The steroid hormone 20-hydroxyecdysone enhances neurite growth of Drosophila mushroom body neurons isolated during metamorphosis. *J. Neurosci*. **18**: 8886–8899.

LEE C.Y., COOKSEY B.A. & BAEHRECKE E.H. 2002: Steroid regulation of midgut cell death during Drosophila development. *Dev. Biol*. **250**: 101–111.

LEVINE R.B. & TRUMAN J.W. 1985: Dendritic reorganization of abdominal motoneurons during metamorphosis of the moth, Manduca sexta. *J. Neurosci.* **5**: 2424–2431.

LOCKSHIN R.A. & WILLIAMS C.M. 1965: Programmed cell death. V. Cytolytic enzymes in relation to the breakdown of the intersegmental muscles of silkmoths. *J. Insect Physiol*. **11**: 831–844.

MANCINI M., NICHOLSON D.W., ROY S., THORNBERRY N.A., PETERSON E.P., CASCIOLA-ROSEN L.A. & ROSEN A. 1998: The caspase-3 precursor has a cytosolic and mitochondrial distribution: implications for apoptotic signaling. *J. Cell Biol.* **140**: 1485–1495.

MARTIN D.N. & BAEHRECKE E.H. 2004: Caspases function in autophagic programmed cell death in Drosophila. *Development* **131**: 275–284.

MEHMET H. 2000: Caspases find a new place to hide. *Nature* **403**: 29–30.

MILLER D.T. & CAGAN R.L. 1998: Local induction of patterning and programmed cell death in the developing Drosophila retina. *Development* **125**: 2327–2335.

MIZOGUCHI A., OHASHI Y., HOSODA K., ISHIBASHI J. & KATAOKA H. 2001: Developmental profile of the changes in the prothoracicotropic hormone titer in hemolymph of the silkworm Bombyx mori: correlation with ecdysteroid secretion. *Insect Biochem. Mol. Biol*. **31**: 349–358.

NARKILAHTI S., PIRTTILA T.J., LUKASIUK K., TUUNANEN J. & PITKANEN A. 2003: Expression and activation of caspase 3 following status epilepticus in the rat. *Eur. J. Neurosci*. **18**: 1486–1496.

OBERHAMMER F., WILSON J.W., DIVE C., MORRIS I.D., HICKMAN J.A., WAKELING A.E., WALKER P.R. & SIKORSKA M. 1993: Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J*. **12**: 3679–3684.

PARK C.W., KIM J.H., KIM K.M., HWANG J.S., KANG S.W., KANG H.S., CHO B.P., YU C.H., KIM H.R. & LEE B.H. 2004: Evidence for brain-derived neurotrophic factor-like neuropeptide in brain of the silk moth Bombyx mori during postembryonic periods. *Peptides* **25**: 1891–1897.

PRUGH J., CROCE K.D. & LEVINE R.B. 1992: Effects of the steroid hormone, 20-hydroxyecdysone, on the growth of neurites by identified insect motoneurons in vitro. *Dev. Biol.* **154**: 331–347.

RAY S.K., SCHAECHER K.E., SHIELDS D.C., HOGAN E.L. & BANIK N.L. 2000: Combined TUNEL and double immunofluorescent labeling for detection of apoptotic mononuclear phagocytes in autoimmune demyelinating disease. *Brain Res. Protoc.* **5**: 305–311.

ROBINOW S., DRAIZEN T.A. & TRUMAN J.W. 1997: Genes that induce apoptosis: transcriptional regulation in identified, doomed neurons of the Drosophila CNS. *Dev. Biol*. **190**: 206–213.

SANDSTROM D.J. & WEEKS J.C. 1996: Novel dual innervation of a larval proleg muscle by two similar motoneurons in the tobacco hornworm, Manduca sexta. *J. Exp. Biol*. **199**: 775–791.

SCHMECHEL D., MARANGOS P.J. & BRIGHTMAN M. 1978: Neurone-specific enolase is a molecular marker for peripheral and central neuroendocrine cells. *Nature* **276**: 834–836.

SCHUBIGER M., WADE A.A., CARNEY G.E., TRUMAN J.W. & BENDER M. 1998: Drosophila EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis. *Development* **125**: 2053–2062.

SCHWARTZ L.M. & TRUMAN J.W. 1982: Peptide and steroid regulation of muscle degeneration in an insect. *Science* **215**: 1420–1421.

STREICHERT L.C. & WEEKS J.C. 1995: Decreased monosynaptic sensory input to an identified motoneuron is associated with steroid-mediated dendritic regression during metamorphosis in Manduca sexta. *J. Neurosci.* **15**: 1484–1495.

STREICHERT L.C., PIERCE J.T., NELSON J.A. & WEEKS J.C. 1997: Segment-specific programmed cell death of identified motoneurons triggered directly by steroid hormones in vitro. *Dev. Biol*. **183**: 95–107.

TERASHIMA J., YASUHARA N., IWAMI M., SAKURAI S. & SAKURAI S. 2000: Programmed cell death triggered by insect steroid hormone, 20-hydroxyecdysone, in the anterior silk gland of the silkworm, Bombyx mori. *Dev. Genes Evol*. **210**: 545–558.

TRUMAN J.W. 1983: Programmed cell death in the nervous system of an adult insect. *J. Comp. Neurol*. **216**: 445–452.

TRUMAN J.W. & SCHWARTZ L.M. 1984: Steroid regulation of neuronal death in the moth nervous system. *J. Neurosci.* **4**: 274–280.

TRUMAN J.W., THORN R.S. & ROBINOW S. 1992: Programmed neuronal death in insect development. *J. Neurobiol*. **23**: 1295–1311.

TRUMAN J.W., TAYLOR B.J. & AWAD T. 1993: Formation of the adult nervous system. In Bate M. & Martinez-Arias A. (eds): *The Development of Drosophila melenogaster.* Cold Spring Harbor Laboratory Press, New York, pp. 1245–1276.

TRUMAN J.W., TALBOT W.S., FAHRBACH S.E. & HOGNESS D.S. 1994: Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during Drosophila and Manduca development. *Development* **120**: 219–234.

TSUZUKI S., IWAMI M. & SAKURAI S. 2001: Ecdysteroid-inducible genes in the programmed cell death during insect metamorphosis. *Insect Biochem. Mol. Biol.* **31**: 321–331.

VULLINGS H.G., LEGERSTEE-OOSTVEEN G.G., DIEDEREN J.H. & JANSEN W.F. 1989: Immunocytochemical demonstration of neuron-specific enolase in neuronal cell bodies of an insect. *Neurosci. Lett.* **104**: 48–52.

WANG X. 2001: The expanding role of mitochondria in apoptosis. *Genes Dev*. **15**: 2922–2933.

WEEKS J.C. & DAVIDSON S.K. 1994: Influence of interganglionic interactions on steroid-mediated dendritic reorganization and death of proleg motor neurons during metamorphosis in Manduca sexta. *J. Neurobiol*. **25**: 535–554.

Weil M., Jacobson M.D., Coles H.S., Davies T.J., Gardner R.L., Raff K.D. & Raff M.C. 1996: Constitutive expression of the machinery for programmed cell death. *J. Cell Biol*. **133**: 1053–1059.

Yakovlev A.G., Ota K., Wang G., Movsesyan V., Bao W.L., Yoshihara K. & Faden A.I. 2001: Differential expression of apoptotic protease-activating factor-1 and caspase-3 genes and susceptibility to apoptosis during brain development and after traumatic brain injury. *J. Neurosci.* **21**: 7439–7446.

Young C., Klocke B.J., Tenkova T., Choi J., Labruyere J., Qin Y.Q., Holtzman D.M., Roth K.A. & Olney J.W. 2003: Ethanol-induced neuronal apoptosis in vivo requires BAX in the developing mouse brain. *Cell Death Differ*. **10**: 1148–1155.

Yu S.Y., Yoo S.J., Yang L., Zapata C., Srinivasan A., Hay B.A. & Baker N.E. 2002: A pathway of signals regulating effector and initiator caspases in the developing Drosophila eye. *Development* **129**: 3269–3278.

Zee M.C. & Weeks J.C. 2001: Developmental change in the steroid hormone signal for cell-autonomous, segment-specific programmed cell death of a motoneuron. *Dev. Biol*. **235**: 45–61.

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