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Induction of *Sarcophaga* Central Nervous System Remodeling by 20-Hydroxyecdysone *In Vitro*[1]

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Proliferation and apoptosis of neural cells were found to be induced simultaneously when larval brains of *Sarcophaga peregrina* were cultured in the presence of 20-hydroxyecdysone (20-HE) for 24 h. The locations of proliferating cells and apoptotic cells in the brainhemispheres were different. The morphology of brains exposed to 20-HE for a short period proceeded to change sequentially when culture was continued for 2 days even in the absence of 20-HE. These changes mainly consisted of enlargement of the brainhemispheres and extension of the interval between two hemispheres, which closely paralleled the morphological changes of brains that occur in the early pupal stage, suggesting that ecdysteroid alone is sufficient to induce the remodeling of the central nervous system of holometabolous insects. Synthesis of a protein with a molecular mass of 66 kDa was shown to be selectively repressed when brains were cultured in the presence of 20-HE.

Key words: apoptosis, cell proliferation, CNS remodeling, 20-hydroxyecdysone, *Sarcophaga peregrina*.

During metamorphosis of holometabolous insects, neural circuits that control feeding and wandering in larva are converted to those that control flight, walking, and reproductive behavior in adult. The adult nervous system is not constructed *de novo* during metamorphosis, but is formed by the remodeling of larval sensory neurons, motoneurons, and interneurons (*1, 2*). Remodeling of neurons is accompanied by region-specific changes in the contour of the CNS. In *Drosophila melanogaster*, a small subset of neuroblasts is known to proliferate even after postembryonic neurogenesis has been completed (*3, 4*). Thus, some larval neurons die during metamorphosis and others persist, and the persisted neurons and newly developed neurons are believed to form adult neural circuits (*5-7*).

The metamorphic changes in the nervous system of insects are probably induced by ecdysteroids, the same as in other tissues. In *Manduca sexta*, neural cells from leg motoneurons prepared from the insect in the early pupal stage have been shown to display enhanced neurite outgrowth when cultured in the presence of 20-HE *in vitro* (*8*). Precise studies using cultured tissues have revealed that the concentration of 20-HE is critical to the proliferation and apoptosis of neural cells during optic lobe neurogenesis in *Manduca* (*9*).

Here we report that when the larval brain of *Sarcophaga peregrina* (flesh fly) is cultured *in vitro* in the presence of 20-HE, it undergoes metamorphic changes mimicking the remodeling of the CNS during metamorphosis. We found that 20-HE alone is sufficient to induce the metamorphic changes in the CNS, suggesting that humoral factor(s) produced elsewhere in response to 20-HE are not needed for the remodeling of the CNS. Once the brains were exposed to 20-HE for a short period, the metamorphic changes in the CNS were proceeded sequentially even in the absence of 20-HE. Although overall protein synthesis in the cultured brains increased significantly in the presence of 20-HE, synthesis of a protein with a molecular mass of 66 kDa was found to be selectively suppressed. Trigger of the induction of CNS remodeling during metamorphosis of *Sarcophaga* might be the turning off of the genes for specific proteins, such as the 66 kDa protein by 20-HE.

MATERIALS AND METHODS

Culture of Sarcophaga Brains In Vitro—Sarcophaga brains were cultured under the same conditions as we previously used to culture *Sarcophaga* imaginal discs (*10-13*). Third instar larval brain (containing brainhemisphere and ventral ganglions) with eye-antenna discs were dissected in Grace's insect medium under a binocular microscope. Individual brains were cultured in 350-500 μl of culture medium at 25°C in a water-saturated atmosphere. Grace's insect medium was used in all experiments and it was modified before use by adding HEPES to a final concentration of 10 mM, adjusting the pH to 6.0 with a 1 M NaOH, and adding final concentrations of 100 units/ml of penicillin G and 0.5 mg/ml of streptomycin. To induce metamorphic changes in the cultured brains, we added 20-HE to a final

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Abbreviations: CNS, central nervous system; 20-HE, 20-hydroxyecdysone; BrdU, 5-bromo-2-deoxyuridine; PBS, phosphate-buffered saline; v/v, volume per volume; TBS, Tris-buffered saline; FITC, fluoresceinisothiocyanate; TCA, trichroloacetic acid.

concentration of 10^{-6} M. Induction of morphological changes began in about 2 days.

Cell Proliferation in the Brain—Cell proliferation was detected according to the protocols of the cell proliferation kit (Amersham-Pharmacia). To detect brain cell proliferation *in vivo*, *Sarcophaga* pupae (1 day after pupation) were each injected with 10 μl of BrdU solution (3 mg/ml) and maintained at 25°C for 2 h. For induction of pupation, third instar larvae were transferred from wet condition to dry condition. Pupation started about 16 h later after dry (*14*). To detect cell proliferation in the cultured brains, brains were cultured for 1 day in the presence or absence of 20-HE and then labeled for 2 h by adding BrdU to the culture medium to a final concentration of 15 μg/ml. Labeled brains were frozen in embedding medium (Tissue Tek, MILES) and sliced into 10 μm sections. These sections were placed on poly-L-lysine-coated slide glasses and fixed for 15 min with 1.5% formaldehyde in PBS(I) (8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.5 mM KCl). They were then washed well with PBS(I) and immersed in 2% H_2O_2 in methanol to inactivate endogenous peroxidase.

After washing with phosphate buffer (40.5 mM Na_2HPO_4, 9.5 mM NaH_2PO_4), the samples were successively treated with anti-BrdU mouse monoclonal IgG2a and peroxidase-conjugated anti-mouse IgG. The samples were then washed well with PBS(II) (81 mM Na_2HPO_4, 19 mM NaH_2PO_4, 100 mM NaCl) and treated with a diaminobenzidine (a substrate of peroxidase) solution to visualize BrdU incorporated into DNA. Finally, the samples were dehydrated with 70% (v/v) ethanol, ethanol and xylene, and then mounted with Canada balsam.

Cell Death in the Brain—Apoptotic cells in the brains were detected by TUNEL (terminal transferase-mediated dUTP-X nick end labeling) method according to the protocols of *In Situ* Apoptosis Detection Kit (TaKaRa). Brain sections were fixed for 15 min with 3.7% formaldehyde in TBS [10 mM Tris-HCl (pH 7.4), 130 mM NaCl, 25 mM KCl, 1 mM EGTA]. They were then washed well with TBS, immersed in 2% H_2O_2 in methanol to inactivate endogenous peroxidase, and then incubated with terminal transferase and FITC-dUTP for 90 min at 37°C. Next, the samples were successively treated with peroxidase-conjugated anti-FITC antibody and diaminobenzidine solution to visualize terminally incorporated dUTP. Finally, the samples were dehydrated with 70% (v/v) ethanol, ethanol and xylene, and then mounted with Canada balsam.

Protein Synthesis in the Brain—Brains were cultured in the presence of a mixture of 3 MBq/ml each of [^{35}S]methionine and [^{35}S]cysteine for 0.5, 1, 3, 6, and 24 h, respectively. The brains were then washed with insect saline (130 mM NaCl, 5 mM KCl, 1 mM CaCl) and homogenized in ice-cold PBS(I) containing protease inhibitors (100 μg/ml leupeptin, 0.1 μg/ml pepstatin, and 1 mM phenylmethanesulfonyl fluoride). Then saturated TCA solution was added to the homogenate to a final concentration of 10% (v/v), the acid-insoluble material was trapped on a glass-fiber filter (Whatman GF/C), and its radioactivity was measured by a liquid scintillation counter (LS 3801, BECKMAN). To identify the protein labeled, the TCA-insoluble fraction was subjected to SDS polyacrylamide gel electrophoresis (*15*) followed by fluorography.

RESULTS

Induction of Metamorphic Changes in Cultured Brain—Significant morphological changes were found to be induced when brains dissected from third instar larvae of *Sarcophaga* were cultured in the presence of 20-HE. These changes were very similar to those detected in the brains of metamorphosing *Sarcophaga*. Essentially no morphological changes were induced when brains alone were cultured (Fig. 1B). However, when they were cultured for 2 days in the presence of 10^{-6} M 20-HE, two typical changes in their morphology were detected: enlargement of the brainhemispheres and extension of the interval between the two hemispheres (Fig. 1C). Thickening of the optic stalks, contraction of the ventral ganglion, and development of eye-antenna discs were also observed. These morphological changes were very similar to those detected in the brains of metamorphosing *Sarcophaga* in the early pupal stage (Fig. 1D).

Induction of the morphological changes depended upon the concentration of 20-HE. The results of two independent experiments to determine the optimal concentration of 20-HE are summarized in Table I. No appreciable morphological changes were induced when the 20-HE concentration was below 10^{-8} M. At 10^{-7} M 20-HE, enlargement of the brainhemispheres was detected, but the interval between the two hemispheres did not extend. Above 10^{-6} M 20-HE, the morphology of the brains became abnormal: eye-antenna complexes were blistered and the brainhemispheres were pressed down by these blistered complexes. The optimal concentration of 20-HE was 10^{-6} M. Almost 100% of the cultured brainhemispheres enlarged and under these conditions, extension of the interval between two hemispheres was detected in 7 of the 20 brain samples cultured.

We found that when brains were exposed to 20-HE for 1 h, their morphological changes were sequentially induced even in the subsequent absence of 20-HE. We first cultured brains in the presence of 10^{-6} M 20-HE for appropriate periods, and then transferred them to 20-HE-free medium and continued the culture for 2 days. As shown in Fig. 2, about 70% of brainhemispheres were enlarged when the brains were first cultured for 1 h in the presence of 20-HE and then cultured without 20-HE. Extension of the interval between the two hemispheres was detected in about 40% of the enlarged brainhemispheres. Subsequently, we found that these changes were induced when the brains were exposed to 20-HE for only 10 min (data not shown). These results suggest that once remodeling of the CNS is triggered by 20-HE, it proceeds sequentially irrespective of the presence of the hormone, and exposure of the brains to the hormone for a few minutes is probably sufficient to trigger it. No appreciable differentiation of eye-antenna and leg discs was induced when they were exposed to 20-HE for even 24 h before transferring 20-HE-free medium.

Cell Proliferation and Cell Death during CNS Remodeling—As the morphological changes in the brains are obvious, it is assumed that cell proliferation and cell death are induced in the brains when they are exposed to 20-HE. To detect cell proliferation during CNS remodeling, we performed immuno-peroxidase staining using a monoclonal antibody against BrdU. For this, we cultured the brains in

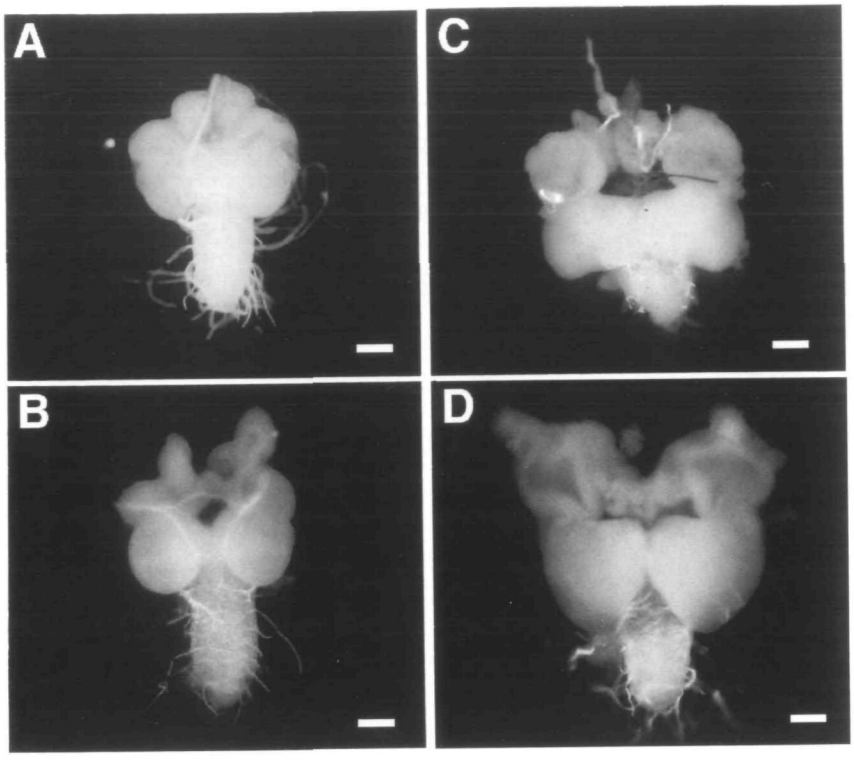

Fig. 1. **Morphological changes in *Sarcophaga* larval brains cultured *in vitro*.** Brains were cultured in modified Grace's insect medium in the presence of 10^{-6} M 20-HE for 4 days. (A) Intact brain complex with eye-antenna discs and ventral ganglion (before culture). (B) Brain cultured in the absence of 20-HE. (C) Brain cultured in the presence of 20-HE. (D) Brain complex of a pupa 2 days after pupation. The bars indicate 200 μm.

TABLE I. **Correlation between the concentrations of 20-HE and morphological changes in the CNS.**

Concentration of 20-HE	Enlargement of brainhemispheres	Extension of the intervals between the two brainhemispheres
0 M (n=20)	0 (0%)	0 (0%)
10^{-8} M (n=9)	0 (0%)	0 (0%)
10^{-7} M (n=12)	11 (92%)	0 (0%)
10^{-6} M (n=20)	20 (100%)	7 (35%)
10^{-5} M (n=13)	13 (100%)[a]	2 (15%)[a]
10^{-4} M (n=9)	9 (100%)[a]	1 (11%)[a]

[a]These samples were abnormal; their eye-antenna complexes were blistered, and the brainhemispheres often seemed to be compressed by the blistered eye-antenna complexes.

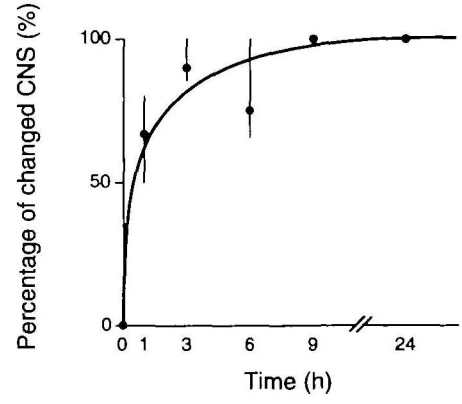

Fig. 2. **Morphological changes in brains induced by pulse exposure to 20-HE.** Brains were first cultured in the presence of 10^{-6} M 20-HE for 1, 3, 6, 9, and 24 h, respectively, and then transferred to ecdysone-free medium. Enlargement of brainhemispheres was assessed 1 week later. In each experiment, 4–8 brains were used. Bars indicate deviations of two independent experiments.

the presence of 10^{-6} M 20-HE for 1 day and labeled them with BrdU. They were then sliced into 10 μm sections, and BrdU-labeled DNA was visualized by immuno-peroxidase staining. As shown in Fig. 3A, signals were detected in the peripheral and central regions of the brainhemisphere, indicating that DNA synthesis, and thus cell proliferation, is induced in these regions. These regions appear to be the optic anlagen, where neurons that form the adult optic lobes develop. No appreciable signals were detected when brains were cultured in the absence of 20-HE (Fig. 3B). To detect cell proliferation in the brains *in vivo*, we injected BrdU directly into pupae aged 1 day after pupation, and labeled the brains for 2 h. As shown in Fig. 3C, cell proliferation was detected in the peripheral and central regions of the brainhemispheres, almost the same as in the brains cultured in the presence of 20-HE. Cell proliferation of laminar precursors was observed only in the pupal brain.

Next, we examined whether cell death occurs during CNS remodeling by examining for the brains apoptosis by TUNEL method. The brains were cultured in the presence of 10^{-6} M 20-HE for 1 day and then sliced into 10 μm sections. The fragmented DNA in each section was labeled with FITC-dUTP by using terminal transferase, and visualized by immuno-peroxidase staining with peroxidase-conjugated anti-FITC antibody. As shown in Fig. 4A, signals of fragmented DNA in apoptotic cells were detected as clusters in the medulla cortex, especially at the boundary between medulla cortex and protocerebrum, and in the region between optic stalk and medulla cortex. In contrast, no appreciable cell death was detected in the brains cultured in the absence of 20-HE (Fig. 4B). When pupal brainhemispheres were examined, apoptotic signals were

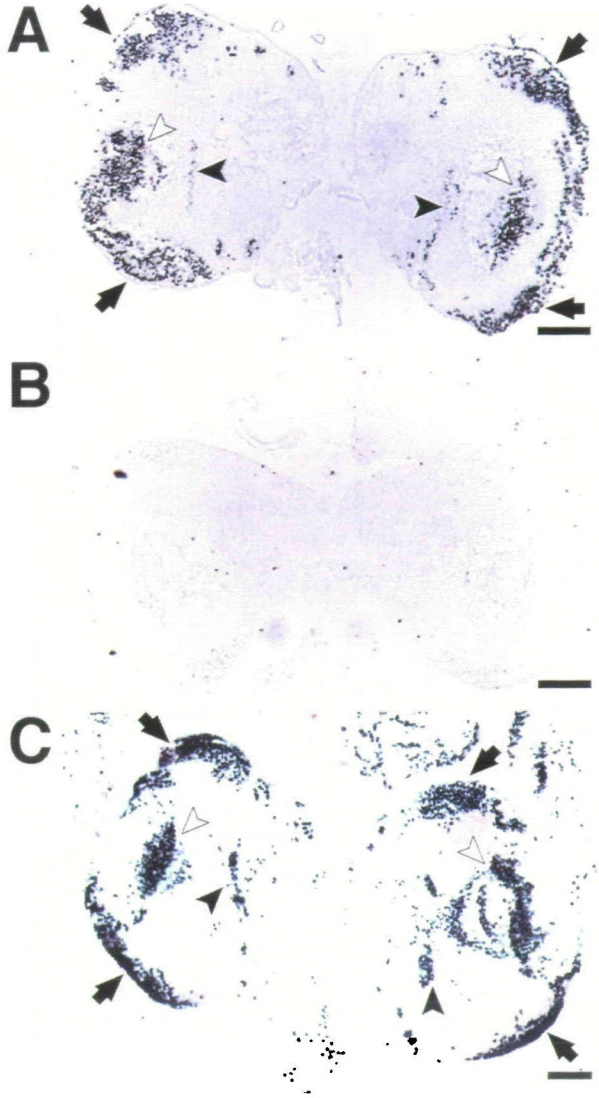

Fig. 3. **Cell proliferation in cultured brains.** The brains were cultured in the presence or absence of 10^{-6} M 20-HE in the medium containing for BrdU for 1 day, and the BrdU incorporated was visualized by immuno-peroxidase staining. (A) Brain cultured in the presence of 20-HE; (B) Brain cultured in the absence of 20-HE; (C) Pupal brain (1 day after pupation). The regions of the brainhemispheres where signals were detected were outer optic anlage (arrow), inner optic anlage (open arrowhead), and neuroblasts of the inner anlage (closed arrowhead). The bars indicate 100 μm.

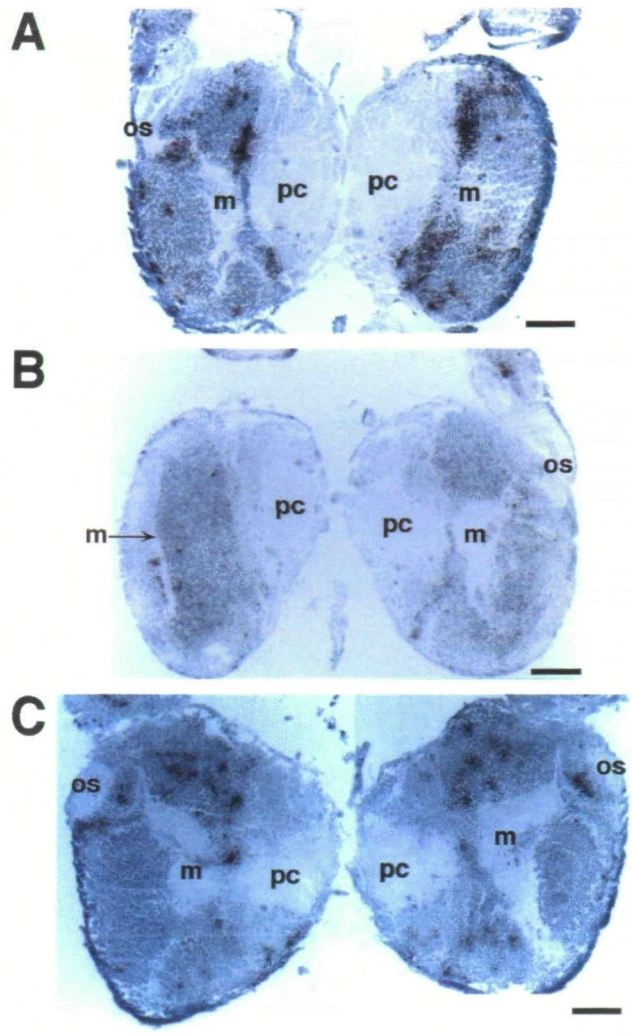

Fig. 4. **Apoptotic cells in cultured brains.** Brains were cultured in the presence or absence of 10^{-6} M 20-HE for 1 day. To detect apoptotic cells, fragmented DNA in the cultured brains was labeled with FITC-dUTP by using terminal transferase, and the labeled DNA was visualized by immuno-peroxidase staining. (A) Brain cultured in the presence of 20-HE; (B) Brain cultured in the absence of 20-HE; (C) Pupal brain (1 day after pupation). m, medulla; pc, protocerebrum; os, optic stalk. The bars indicate 100 μm.

detected almost in the same regions where cell death was induced *in vitro* (Fig. 4C).

These results indicate that both cell proliferation and cell death are induced simultaneously in restricted regions in the brains when they were exposed to 20-HE, but the regions where cells proliferate and those where cells die are clearly different. Extensive changes in the nerve cell population are required for remodeling of CNS, and this may be achieved by the cell proliferation and cell death provoked by 20-HE.

Protein Synthesis in the Cultured Brain—It became evident that the nerve cell population changes significantly when brains were cultured in the presence of 20-HE for 24 h. To gain more insight into the molecular mechanisms of CNS remodeling, we analyzed protein synthesis in the brains by culturing them in the presence or absence of 10^{-6} M 20-HE and labeling them with a mixture of [^{35}S]Met and [^{35}S]Cys for 0.5, 1, 3, 6, and 24 h, respectively. The amount of acid-insoluble radioactivity incorporated into the brains did not change appreciably, irrespective of the presence of 20-HE, when incubation time was below 3 h, but thereafter it increased significantly, as shown in Fig. 5. Much less radioactivity was incorporated in the absence of 20-HE, indicating that protein synthesis in the brain was enhanced in response to 20-HE.

We analyzed the labeled proteins by SDS polyacrylamide gel electrophoresis followed by fluorography to examine whether synthesis of specific proteins is enhanced in the presence of 20-HE. As shown in Fig. 6, the electrophoretic profiles of proteins labeled in the presence and absence of 20-HE were almost the same, except for a 66-kDa protein,

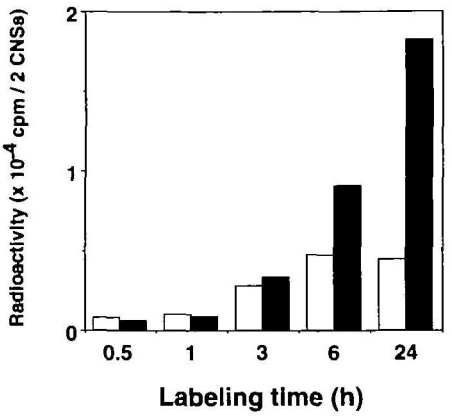

Fig. 5. **Protein synthesis in the cultured brains.** Brains were cultured in the presence or absence of 10^{-6} M 20-HE for 0.5, 1, 3, 6, and 24 h, respectively, in medium containing a mixture of [^{35}S]-methionine and [^{35}S]cysteine. Acid-insoluble radioactivity incorporated into the brains was measured. Open bars, brains cultured in the absence of 20-HE; closed bars, brains cultured in the presence of 20-HE.

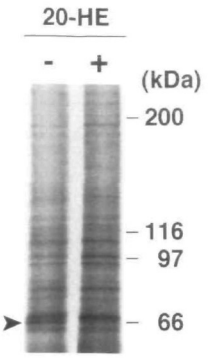

Fig. 6. **SDS polyacrylamide gel electrophoresis of brain proteins.** Brains were cultured in the presence or absence of 10^{-6} M 20-HE 24 h, in medium containing a mixture of [^{35}S]methionine and [^{35}S]cysteine. Labeled proteins were analyzed by SDS polyacrylamide gel electrophoresis followed by fluorography. The gel was calibrated with various molecular mass markers shown in kDa on the right. The arrowhead points to a protein with a molecular mass of 66 kDa.

which is indicated by an arrow. This protein seemed to be synthesized only in the absence of 20-HE, and the negative effect of 20-HE on the expression of this 66-kDa protein gene may be crucial for subsequent remodeling of the CNS.

DISCUSSION

The neural circuits of holometabolous insects must change as they develop from larvae to adults. As the first step in analyzing the molecular mechanisms of the remodeling of insect neural circuits, we established an *in vitro* culture system of *Sarcophaga* brains in which they undergo morphological changes that mimic those that occur *in vivo*. The morphological changes in the brain were induced by 20-HE alone and its concentration was very critical. We assessed the morphological changes in the brains with two parameters: enlargement of the brainhemispheres and extension of the interval between the two brainhemispheres. At 10^{-6} M 20-HE, 100% of the cultured brainhemispheres enlarged, but the intervals between enlarged hemispheres did not always extend under these conditions, with interval-extension usually being detected in about 50% of the enlarged brains. Thus, enlargement of the brainhemispheres probably depends on 20-HE, but interval extension requires another factor whose activity is difficult to control in our experimental system. It took at least 2 days to induce obvious morphological changes in the cultured brains, but 20-HE was found to be needed only to initiate the remodeling reaction. Exposure of the brains to 20-HE for 1 h (perhaps less than that) was sufficient to induce subsequent morphological changes, which subsequently proceeded sequentially even in the absence of 20-HE. Exposure of imaginal discs to 20-HE for even 24 h did not induce appreciable differentiation. This suggests that there may be a brain-specific ecdysone receptor that rapidly responds to added 20-HE.

Although no appreciable external morphological changes in the brain were detected during first 24 h, the brains was found to change extensively internally on the cellular and molecular levels. We detected cell proliferation and cell death caused by 20-HE during this period. Cell proliferation occurred in the peripheral and central regions of the brainhemispheres, whereas cell death was detected mainly at the boundary between the medulla cortex and protocerebrum and the region between the optic stalk and medulla cortex. These regions are considered necessary for the formation of the adult visual system (*16*). The proliferating cells are supposed to form adult optic lobes. The apoptotic cells may be destined to be excluded because they are unnecessary for the formation of the adult visual system. Moreover, these cellular responses depend entirely upon 20-HE. In *Drosophila*, three ecdysone receptors (EcR) are known (*17*), and these receptors act as a homo and/or hetero dimer (*18*). Combinations of these receptors are thought to be crucial for the selective activation of the target genes of ecdysone. Furthermore, the neurons expressing one of the EcR isoforms, EcR-A, undergo rapid degeneration at the adult emergence as ecdysteroids decline (*19*). Therefore, the combinations of ecdysone receptors and molecules interacting with them in the proliferating cells and apoptotic cells are probably different.

We detected a protein with a molecular mass of 66 kDa that was synthesized only by the brain cultured in the absence of 20-HE. Its synthesis seems to be repressed in the presence of 20-HE. As the expression and repression of this protein are very clear, analyzing it might provide a clue to the molecular mechanism of the remodeling of CNS. There must be other differences in protein synthesis between the two brain samples, but they could not be detected by the analytical procedure we employed. More precise analysis of proteins whose synthesis is affected by 20-HE is required to reveal the molecular mechanism of CNS remodeling.

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