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Effects of 20-Hydroxyecdysone and Serotonin on Neurite Growth and Survival Rate of Antennal Lobe Neurons in Pupal Stage of the Silk Moth *Bombyx mori in vitro*

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ABSTRACT—Effects of 20-hydroxyecdysone and serotonin on the morphological development and the survival of antennal lobe neurons from day-2 pupal brains of the silk moth *Bombyx mori* were investigated *in vitro*. Four morphologically distinct neuronal types could be identified in the cultured antennal lobe neurons: unipolar, bipolar, multi-polar and projection neurons. Antennal lobe neurons in culture with 20-hydroxyecdysone and serotonin showed different patterns of the morphological development from those described in *Manduca sexta*. Projection neurons extend their neurites remarkably by 20-hydroxyecdysone in *B. mori*, but there is no extension from antennal lobe neurons in *M. sexta*. Multi-polar neurons conspicuously increase only formation of new branches from their primary neurites by serotonin in *B. mori,* but there are both extension and branching of the neurites in *M. sexta*. On day-5, antennal lobe neurons in lower titers of 20-hydroxyecdysone had significantly higher survival rates than those in higher titers. Neurons cultured for 7 days at different levels of 20-hydroxyecdysone generally showed significantly lower survival rates than neurons cultured for 5 days under the same conditions.

Key words: neuron culture, 20-hydroxyecdysone, serotonin, neurite growth, silk moth

INTRODUCTION

The antennal lobe (AL, or deutocerebrum of the insect adult brain) is the first central olfactory center which consists of neuropil with the glomeruli, somata gathered in 3 distinct and separate groups lying in lateral, medial and anteroventral AL, and sensory axons from the antennae in the sphinx moth *M. sexta* (Christensen and Hildebrand, 1987; Homberg *et al*., 1988, 1989; Hayashi and Hildebrand, 1990).

In vivo, the population of neurons from *M. sexta* AL can be subdivided into two classes (Matsumoto and Hildebrand, 1981; Homberg *et al*., 1988): projection neurons and local interneurons. These neurons in all stages of pupal development from *M. sexta* could be classified into six different neuronal types with distinctive and stable morphology *in vitro*: radial, proximal branching 1 and 3, symmetrical, fuzzy compact (FC) and rick rack (RR) neurons (Oland and Hayashi, 1993; Mercer *et al*., 1995). The first three neurons are a group of projection neurons *in vivo*, whereas the remaining three neurons belong to the category of amacrine local interneurons.

The glomerular neuropil is the site of interactions among primary sensory afferent neurons, local interneurons, projection neurons, and a small number of centrifugal neurons that enter the AL from other sites in the brain (Christensen and Hildebrand, 1987; Homberg *et al*., 1989; Boeckh and Tolbert, 1993; Mercer *et al*., 1995).

Various hormones, including 20-hydroxyecdysone (20-HE) (Zee and Weeks, 2001), juvenile hormone (Vanhems *et al*., 1990; De Loof *et al*., 2001; Isshiki *et al*., 2001; Mizoguchi, 2001), and allatotropin and allatostatin (Lee, 2001; Park *et al*., 2001; Park and Lee, 2001; Park *et al*., 2002; Teal,

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2002), are associated with events of metamorphic adult development in insects. In particular, 20-HE, a derivative of ecdysone which is released from the insect prothoracic gland, is responsible for inducing and coordinating the events of metamorphic adult development (Prugh *et al*., 1992; Oland and Hayashi, 1993; Streichert *et al*., 1997), whereas serotonin (5-hydroxy- tryptamine, 5-HT) is released from various neurons of the insect central nervous system and then involved to promote the morphological development of some insect neurons (Mercer *et al*., 1995).

Neurons in the AL of developing *M. sexta* brain undergo a period of extensive outgrowth of process and branching that coincide temporally with a rising titer of 20-HE (Oland and Hayashi, 1993). Morphological changes of certain motoneurons and sensory neurons in the thoracic and abdominal ganglia of *M. sexta* are induced by 20-HE (Weeks and Truman, 1985; Levine and Weeks, 1989; Witten and Levine, 1991). Motoneurons dissociated from the ganglia of metamorphosing animals respond directly to the presence of 20-HE in the medium with the extensive outgrowth of process (Prugh *et al*., 1992).

AL neurons dissociated from the stage-5 pupal brain of *M. sexta* respond only weakly, if at all, to 20-HE *in vitro* (Oland and Oberlander, 1994b). They do, however, show a greater total outgrowth and branching when they are exposed *in vivo* to antennal sensory axons. In AL neuron cultures of stage-5 to stage-18 pupal brains 5-HT enhances the growth of some neurons, although it has no effect on their survival (Mercer *et al*., 1995).

The morphological development of the *B. mori* AL neurons *in vitro* was greatly different from those described previously in *M. sexta* (Oland and Hayashi, 1993; Mercer *et al*., 1995). AL neurons of *M. sexta* do not extend the length of their neurites in culture with 20-HE, whereas in culture with 5-HT they not only extend their neurites but also enhance branching of primary neurites. In culture of *B. mori* AL neurons *in vitro*, however, 20-HE induced a remarkable neurite extension of specific AL neurons and 5-HT enhanced only a rise in branch number of specific AL neurons. In this paper these results are described in detail.

MATERIALS AND METHODS

Animals

Eggs of the silkworm *Bombyx mori* (Lepidoptera, Insecta), which were supplied from the National Institute of Agricultural Science and Technology (Suwon, Korea) and hatched in the laboratory, were reared on an artifical diet (purchased from Chongju silk museum, Korea) with a photoperiod regimen of 12hr light/12h dark at 27–28°C and 60–70% relative humidity. Silkworms began the metamorphosis to pupae at the end of the fifth (final) instar. Soon after the molt of the larvae into pupae, the metamorphosis of the pupae into adults began with the initiation of development into an adult brain, and the development of the adult brain proceeded through all the pupal stages.

Dissection of brain and dissociation of neurons

The brains were isolated by dissection with aseptic technique from day-2 pupae anesthetized by chilling on ice for about 15 min and transferred to a sterile culture saline (see below) in an uncoated 35-mm NUNC^{TM} culture dish. Prior to dissection, bilateral antennal anlagens were removed from all pupae within 6 hr after the larval-to-pupal molt, as previously described in *M. sexta* (Oland and Tolbert, 1987). AL neurons dissociated from day-2 pupal brains were used for a morphological classification of neuronal cell types, an investigation of effects by 2-HE and 5-HT, and analysis of survival rates in culture experiments.

AL was dissected from the brain using the meshwork of the tracheae that forms a line of demarcation between the AL and the adjacent brain tissue as a landmark, and then the sheath was eliminated (Hayashi and Hildebrand, 1990). Immediately after dissociation of the AL, all neurons of the lateral, medial and anteroventral groups were removed for culture.

To obtain individual neuronal cells, dissociated AL was incubated at room temperature for 5 min in 2 ml of Hank's Ca^{2+}- and Mg^{2+}-free balanced salt solution (GIBCO) containing 0.5 mg/ml collagenase (Worthington Biochemical Corporation, type I) and 2 mg/ml Dispase (Boehringer Mannheim, grade II, protease neutral).

Medium

Culture saline. Saline used for dissection of the brain/antennal lobe and dissociation of neurons was prepared as previously described (Oland and Hayashi, 1993). Culture saline consisted of 149.9 m*M* NaCl, 3 m*M* KCL, 3 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 10 m*M* *N*-tris-(hydroxymethyl)-methyl-2- aminoethane sulfonic acid, 11 m*M* D-glucose, 5 g/l lactalbumin hydrolysate (GIBCO), 5 g/l TC yeastolate (Difco), 10% fetal bovine serum (FBS, Hyclone), 100 units/ml penicillin, 100 μg/ml streptomycin, pH 7.36 mOsm.

Culture medium. Supplemented-L15 (Leibovitz's L-15 medium) consisted of 500 ml of L15 which 10% FBS, 185 mg - ketoglutaric acid, 200 mg D-(–)-fructose, 350 mg D-glucose, 335 mg D-L-malic acid, 30 mg succinic acid, 1.4 g TC yeastolate, 1.4 g lactalbumin hydrolysate, 0.01 mg niacin, 30 mg imidazole, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 ml stable vitamin mix were added. A 5-ml stock solution of stable vitamin mix consisted of 15 mg aspartic acid, 15 mg cystine, 5 mg -alanine, 0.02 mg biotin, 2 mg vitamin B_{12}, 10 mg inositol, 10 mg choline chloride, 0.5 mg lipoic acid, 5 mg *p*-aminobenzoic acid, 25 mg fumaric acid, 0.4 mg coenzyme A, 15 mg glutamic acid, and 0.5 mg phenol red. The pH was adjusted to 7.0, and the osmolarity to 360 mOsm. All media were sterilized by filtration through a 0.22- μm filter (MILLEX-GS, MILLPORE) prior to use and stored at 5°C.

Primary culture

AL tissue dissociated as described above was then dispersed by trituration with a fire-polished Pasteur pipette. The enzyme action was ended by centrifugation at 1000 rpm for 10 min, first through 1 ml of culture saline and then through 1 ml of Leibovitz's L-15 medium. After removal of the supernatant, the pellet was resuspended in a new Leibovitz's L-15 medium by gentle flicking of the tube bottom and gentle aspiration with a 1 ml pipette. The suspension was then plated onto an uncoated 35-mm NUNC^{TM} culture dish with an additional 2 ml of Leibovitz's L-15 medium. The cultures were kept at 28°C in a 60–70% humidified incubator with normal air, as previously described (Hayashi and Hildebrand, 1990). Throughout the entire experiment, specific cell location in the culture dish was photographed with an inverted microscope.

Anti-HRP staining

It is known in *Drosophila* embryo that antibodies against horseradish peroxidase (anti-HRP) recognize a neural-specific carbohydrate moiety expressed by at least 17 membrane glycoproteins (Jan and Jan, 1982; Snow *et al*., 1987; Kreissl and Bicker, 1992). For labeling of neurons with the anti-HRP antibodies the culture medium was replaced by Ringer solution and subsequently fixed by

4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature. After the cells were rinsed several times in PBS, incubated in 10% methanol/1% H_2O_2 for 10 min in order to inactivate endogenous peroxidase activity, and rinsed again in PBS, the anti-HRP serum was applied to the cultured cells at a dilution of 1:1000 in PBS containing 2% normal goat serum and 0.1% Triton X-100. Incubation was performed at 4°C overnight. The cells were rinsed several times in PBS, and immunoreactivity was visualized by an indirect peroxidase method with a secondary HRP-coupled goat anti-rabbit IgG diluted 1:200 for one day. The cells were then rinsed in PBS, washed in 0.05M Tris-HCl (pH 7.6), and transferred into 0.03% DAB and 1% H_2O_2 in 0.5M Tris-HCl) for 30 min at 4°C. The cells were transferred into a fresh Tris-HCl buffer and photographs for the labeled cells were taken with a Nikon microscope using phase contrast optics. Photographs of the labeled AL cells were used as reference data to determine whether culturing AL cells were neurons or non-neuronal cells (mainly glial cells).

Exposure of 20-HE and 5-HT

AL neurons of the brains from day-2 pupae, in which the AL has no ingrowth of sensory axons from the antenna (Kent *et al*., 1987; Oland and Hayashi, 1993; Oland and Oberlander, 1994a, 1994b; Ai *et al*., 1998; Hansson and Anton, 2000), were grown in the culture media to which 0.0, 0.2, 1.0 or 10.0 μg 20-HE/ml (Sigma H-5142) had been added, respectively. In the hormone manipulation experiments, neurons were exposed to four doses of 20-HE that corresponded to the range of titers found in the hemolymph of developing insects (Oland and Hayashi, 1993). To investigate the responsiveness and neurite growth of the neurons, these levels of 20-HE were maintained by both changes of the culture medium and new adding of 20-HE every day for 1 to 15 days. The total neurite growth of the neurons was compared with that of the neuron grown for the same length of time in the control culture medium without 20-HE.

In order to determine the effect of 5-HT on neurite growth, AL

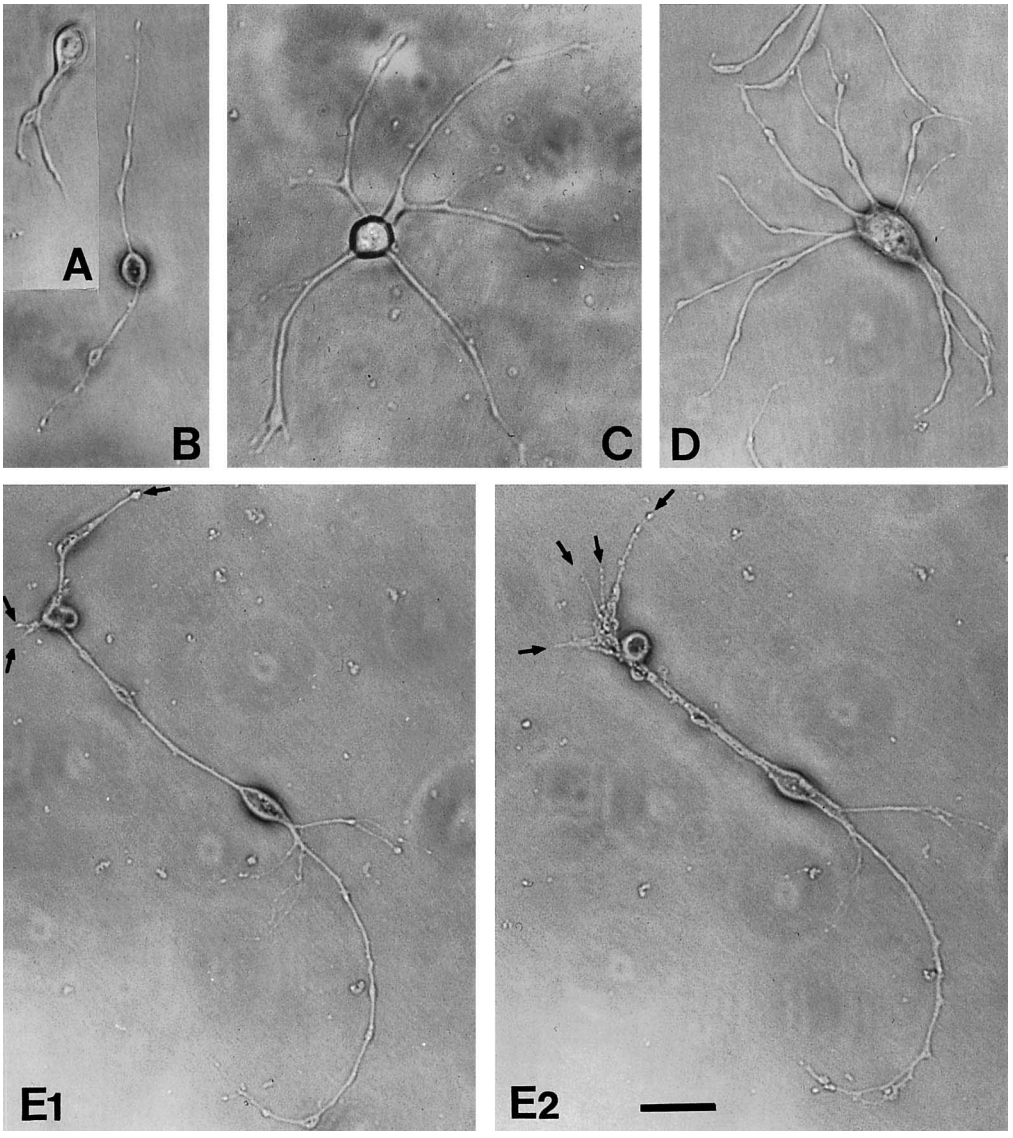

Fig. 1. Major neuronal features in primary culture made from AL of the day-2 pupal brain of *B. mori*. Magnification of all photographs is same as in Fig. 1E2. A: The unipolar neuron after 2 days in culture. B: Bipolar neuron after 3 days in culture. C and D: Two multi-polar neurons in 4-day (C) and 5-day (D) cultures. E1 and E2: The comparison of the same neuron, which appears to be a projection neuron with a longer process, in 4-day (E1) and 9-day (E2) cultures. The neuron has more branched and extended telodendria (small arrows) in 9 days culture (E2) than in 4 days culture (E1). Scale bar in E2 indicates 50 μm.

neurons of day-2 pupal brain were treated with 5-HT in a normal culture medium. Neuronal culture with 5-HT was carried out as previously described (Oland and Hayashi, 1993; Mercer *et al*., 1995). The culture media in all dishes were changed with a daily addition of 50 μ*M* 5-HT, as well as at an initial concentration of 50 μ*M*. 5-HT was prepared immediately prior to use. Because of the assumption that the concentration of 5-HT decreases with time and the byproducts of 5-HT decomposition affect cell growth, in a dish in each of more than ten successive experiments the culture was replaced daily with a medium transferred from cells treated 22 to 24 hr earlier with 5-HT. Data obtained from treatment of 5-HT in the culture of AL neurons was compared with those from the neurons grown for the same length of time in the control culture medium without 5-HT.

Measurement of neurite growth and statistical analysis

Photographs of culturing projection and multi-polar neurons were obtained for the measurements of the extended length of neurite and/or counts of increased branching of neurite. After 1 to 15 days in culture, the neurite growth (the extended length and increased branches of neurite) of cultured neurons were analysed with photographs taken periodically from the culturing neurons with an inverted microscope. Because contacts among the culturing cells made it difficult to determine the length and origin of some neurites, only cells virtually free of contact with other neurites were analyzed. To prevent the occurrence of overlapping growth to the cells, they were plated with a low density of 1 animal per dish. Data from cultures of the neurons in primary culture and with 20-HE and 5-HT were acquired for 3 years, and the full data set contains measurement from over 300 neurons.

A statistical analysis was performed using the SPSS10.0 program for correlated or independent means. Data were expressed as means ±95% confidence intervals of the difference.

Neuronal survival

Survival rates of some AL neurons at 5 and 7 days in culture were investigated according to those described by Oland and Hayashi (1993). Neurons were plated onto a culture dish in which the bottom of the well had been made with a gridded cover slip (Belco) to permit sequential photographs of the same regions of the dish. For each of the 20-HE doses, photographs were taken from at least five randomly chosen grid squares in each of four to six dishes from each of three experiments. Photographs were taken on day-1 (24 hr after plating, to ensure that most of the cells, which have been injured by the dissociation procedure, had died), day-5 and day-7. Neurons were included in the day-1 count if they appeared with an apparent external feature and began the extension of process. Survival rates on day-5 and day-7 in culture were calculated as a percentage of the neurons present on day-1. Survival rates for each dose were compared using the method of

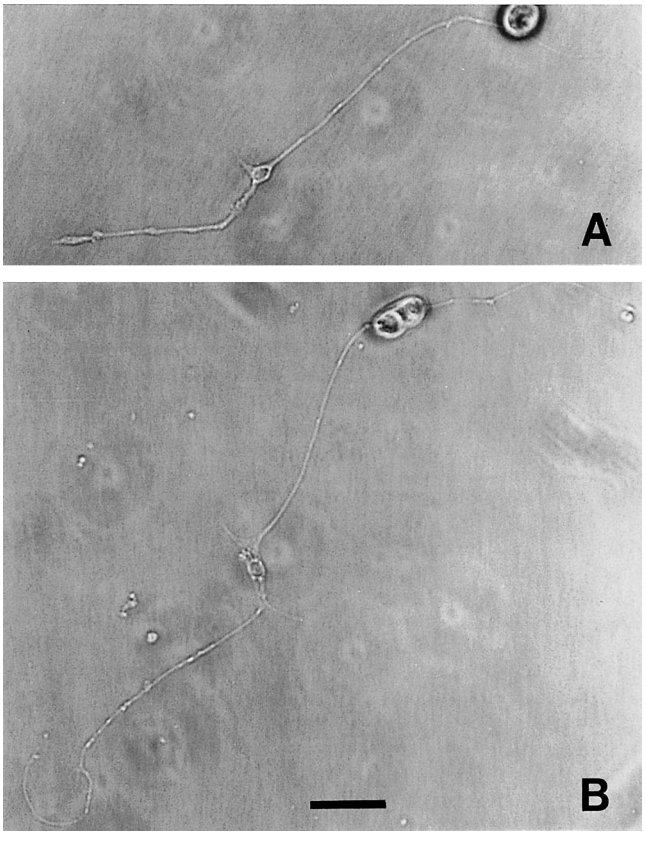

Fig. 2. The comparison of a developing AL neuron of the day-2 pupal brain in 2 and 4 days cultures with 10.0 μg 20-HE/ml, respectively. A: The neuron in 2 days culture with 20-HE. Magnification is the same as in Fig. B. B: The neuron in 4 days culture with 20-HE. The projection neuron has much longer neurites after 4 days culture than after 2 days in culture. Scale bar indicates 50 μm.

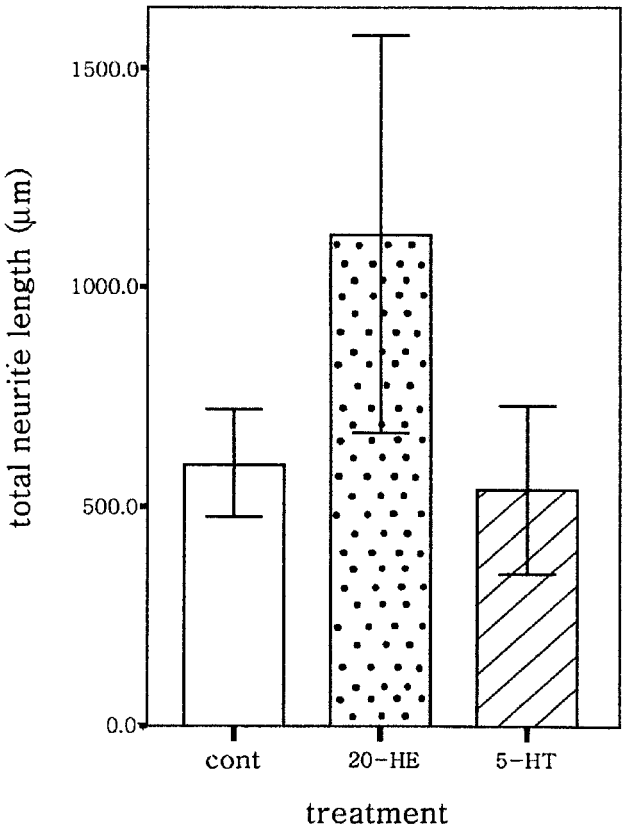

Fig. 3. The mean total length of neurite of AL projection neurons in cultures in which the neuron was respectively exposed to 20-HE (10.0 μg/ml) and 5-HT (50 μ*M*) with control culture of the neuron not exposed to either of them. These results were originated from the data measured during day-4 to day-7 in culture. 20-HE remarkably stimulates the projection neurons to extend its neurite at a mean extension rate of 22.3 μm/day in culture of day-4 to day-7, in comparison with control culture (5.2 μm/day) or culture with 5-HT (4.9 μm/day) (both $p<0.05$). These statistical analyses for measuring new extension of a primary neurite were made from 671 projection neurons and similar numbers of other three AL neuronal types. The error bars show mean ±95% confidence intervals.

Adjusted Significance Levels for proportion (Ryan, 1960).

RESULTS

Neuronal types in AL primary cultures

AL neurons which were dissociated from day-2 pupal brains could be seen as growing into morphologically maturing neurons in primary cell cultures for up to 2 weeks. Four neuronal cell types with a distinctive and stable morphology appeared in cultures made from the AL of day-2 pupae (Fig. 1). Unipolar neurons could be recognized easily during these culture experiments (Fig. 1A). Some of these unipolar neurons, which were shown to be the most frequent type, had several processes to grow fully in the primary culture. The second type of AL neurons was the bipolar neurons (Fig. 1B), which could be grown into other types of neurons in culture, such as projection neurons. Multi-polar neurons had several typical processes extended from the cell bodies (Fig. 4A). Multi-polar neurons did not always exhibit long processes characteristic of an axon of a projection neuron (Figs. 1C and 1D). Some of them were also found to develop various neurites from aggregates of their cell bodies as seen also in Figs. 1C and 1D. This type was found to be an extremely small number in culture. The fourth type of AL neurons was the projection neuron. This neuron had a longer axon-like structure than the other neurons, as well as abundantly arborizing telodendria (Figs. 1-E1, 1-E2, small arrows). This neuron had also several numbers of dendrite-like processes.

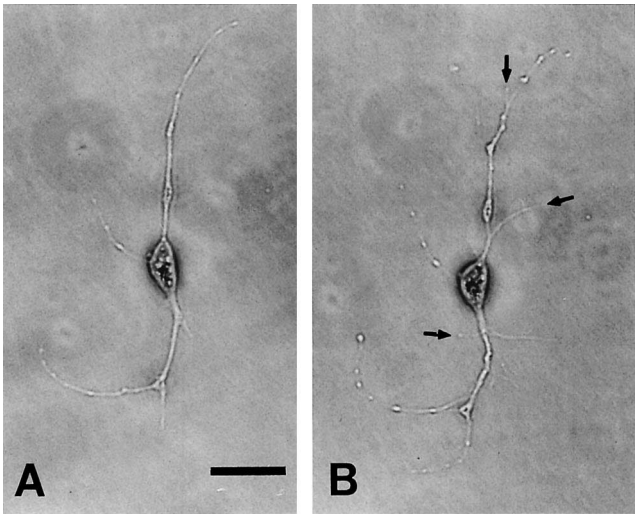

Fig. 4. The comparison of an AL neuron of the day-2 pupal brain in 3 days and 5 days cultures with concentration of 50 μ*M* 5-HT, respectively. A: The multi-polar neuron in 3 days culture with 5-HT. Scale bar indicates 50 μm. B: The multi-polar neuron in 5 days culture with 5-HT. These multi-polar neurons have more branches on the primary neurites in 5 days culture than in 3 days culture and show no extension of neurite. Magnification is the same as in Fig. 4A.

Effect of 20-HE on neuronal growth

The doses used in this experiment to investigate the effect of 20-HE on neuronal growth originated from the physiological range measured in the hemolymph of a moth (Oland and Hayashi, 1993).

Morphological photographs of AL neurons cultured with 20-HE were obtained from four types of AL neurons, but the positive effect of 20-HE could be found only in the projection neuron of the four types of AL neurons (Fig. 2). Statistical data obtained from the morphological data of our experiments also showed that different doses of 20-HE had a positive effect on the morphological growth of the projection neurons (Fig. 3), with no positive responsiveness in the remaining three types to 20-HE. Positive responsiveness of the projection neurons was indicated as a remarkable exten-

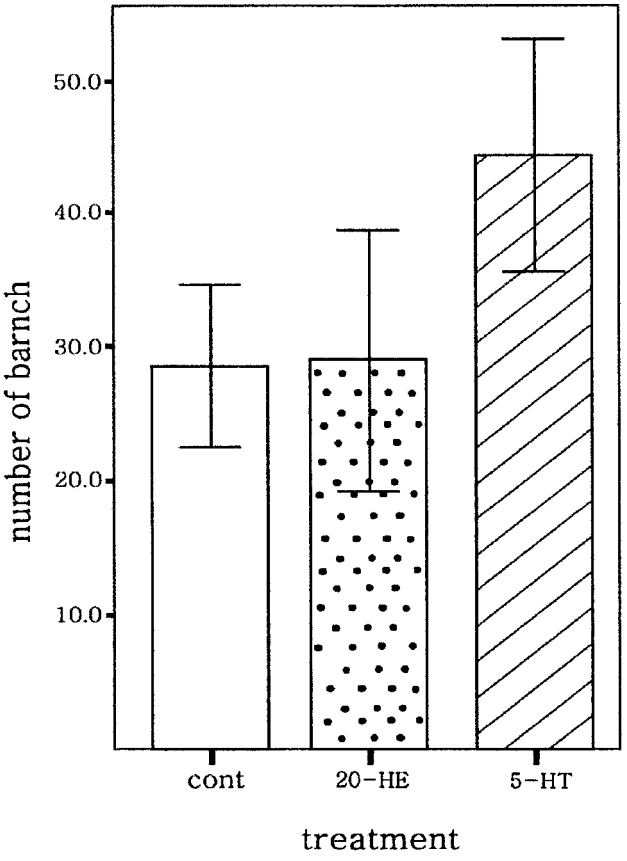

Fig. 5. The mean number of branches of AL multi-polar neurons in cultures in which the neuron was respectively exposed to 5-HT (50 μ*M*) and 20-HE (10.0 μg/ml), with control culture of the neuron not exposed to any of them. These results were originated from the data investigated during day-5 to day-7 in culture. 5-HT remarkably stimulates the multi-polar neurons to form new branches (small arrows in Fig. 4) from the primary neurites at a rate of 5.2/day in culture of day-5 to day-7, in comparison with 1.1/day in control culture and 1.3/day in culture with 20-HE ($p<0.01$). However, there is no significant difference in outgrowth of the new branches between control culture and culture with 20-HE. These statistical data for counting newly extended neurites from a primary neurite were investigated in 648 multi-polar neurons and similar numbers of other three AL neuronal types. The error bars show mean ±95% confidence intervals.

sion of their neurites (Fig. 2). As shown in Fig. 3, the mean total neurite of projection neurons after treatment of 20-HE was significantly extended, but significant neurite extension could be found in neither the control group of the AL neurons without treatment of 20-HE nor experimental groups of the AL neurons with treatment by 5-HT. These statistical analyses for measuring newly extended neurite of a principal neurite were performed from 671 projection neurons and similar numbers of other three AL neuronal types.

Actually, the most remarkable and rapid extension of the neurite from projection neurons occurred from day-4 to day-7 cultures (days of the most extension rate) with 10.0 μg 20-HE/ml, with slower and weaker extension rates in less concentrations and later days. A mean extension rate of a principal neurite in day-4 to day-7 cultures with 10.0 μg 20-HE/ml was 22.3 μm/day, but it was 5.2 μm/day in control culture and 4.9 μm/day in culture with 5-HT.

Effect of 5-HT on neuronal growth

In order to trace which types of the AL neurons showed the neurite growth with 5-HT treatment and which types of the neurite growth occurred in the cultured AL neurons, four types of AL neurons were cultured with 50 μ*M* of 5-HT. As seen in Fig. 4, an apparent neurite growth appeared in the multi-polar neurons, with no positive responsiveness to 5-HT in the remaining three types of neurons. 5-HT stimulated the multi-polar neurons to increase the number of branches (small arrows) projecting from the primary neurite. Statistical data, made from 648 multi-polar neurons and similar numbers of other three AL neuronal types cultured with 5-HT, verified that multi-polar neurons had a significant rise in the number of neurite branches (Fig. 5). As a result of statistic treatment of the AL neurons cultured with 20-HE, a significant increase of branches could not be detected from more than ten experiments.

Actually, the most number of branches of the principal neurites from multi-polar neurons was newly extended in day-5 to day-7 cultures (days to form the most number of branches) at 50 μ*M* of 5-HT, in comparison with a new formation of less number in later days. A mean number of the neurite branch newly extended from a multi-polar neuron in day-5 to day-7 cultures was 5.2/day, but it was 1.1/day in control culture and 1.3/day in culture with 20-HE.

Effect of 20-HE on neuronal survival

The figures of 20-HE (0.0, 0.2, 1.0, 10.0 μg/ml) used for the survival rates of AL neurons were derived from data in the moth (Warren and Gilbert, 1986; Oland and Hayashi, 1993). A dose of 0 μg/ml was included in this study to determine whether neuron survival depended on the presence of 20-HE. The 10 μg/ml dose was also included to ensure that the neurons were exposed to a supramaximal dose.

Survival rates for AL neurons dissociated from day-2 pupal brain were determined by comparing the number of

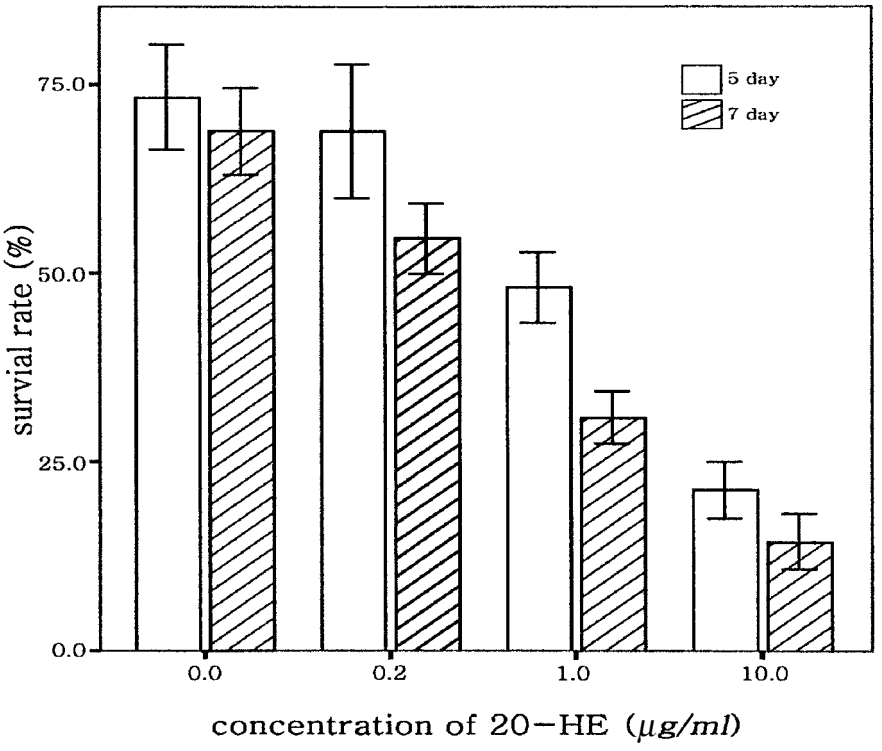

Fig. 6. Survival rates of day-5 and day-7 culture groups of AL neurons exposed to different levels of 20-HE (0.0, 0.2, 1.0, 10.0 μg/ml). The day-7 cultured neuronal groups show significantly lower survival rates than the day-5 cultured neuronal groups at 0.2 μg/ml ($p<0.005$), 1.0 μg/ml ($p<0.001$) and 10.0 μg/ml ($p<0.01$). These statistical analyses for investigating the survival rates were obtained from 853 AL neurons. The error bars show mean ±95% confidence intervals.

neurons at day-5 and day-7 after plating with the number of neurons that had been present in the day-1 culture. Only neurons with processes were included in the day-1 count. No attempt was made to examine survival rates of specific neuronal types for two reasons: these types comprised only a small proportion of the neurons in each low-density culture and neuronal types could not be identified by their characteristic shape in the day-1 culture.

Surviving AL neurons continued to decrease in number after 5 days in culture with 20-HE. At day-5, neurons cultured in a 0- or 0.2-μg/ml 20-HE titer had significantly higher survival rates than those cultured in a 1- or 10-μg/ml titer (p<0.05), as shown in Fig. 6. There were no significant differences in survival between the 0- and 0.2-μg/ml doses. Except for two neuronal groups cultured with the 0-μg/ml dose, day-7 cultured neuronal groups exhibited significantly lower survival rates than day-5 neuronal groups at 0.2-μg/ml (p<0.005), 1.0-μg/ml (p<0.001), and 10.0-μg/ml (p<0.01).

These statistical analyses for investigating the survival rates were made from 853 AL neurons.

DISCUSSION

Neuronal types in AL primary cultures

AL neurons of only day-2 pupal brains from *B. mori* were classified into four types in the culture: unipolar, bipolar, multi-polar and projection neurons. In *M. sexta*, however, 6 AL neuronal types could be identified from metamorphosing brains from all stages (18) of the pupal development, and projection neurons typically undergo tufted arborization in one or a few glomeruli and send an axon to higher-order centers in the brain (Oland and Oberlander, 1994a, 1994b). Local interneurons typically form tufted arbors in most if not all glomeruli and do not have axons leaving the lobe.

Effect of 20-HE on neuronal growth

Data obtained from this investigation strongly suggested that steroid hormone, 20-HE, showed strong effects to extend the neurites of a specific cultured AL neuronal type from *B. mori*. This fact does not support experimental results described from culture of the *M. sexta* AL neurons with 20-HE which does not extend the length of their neurites. Most of the projection neurons in the AL at day-2 pupal period, in which ingrowth of sensory axons from antenna to the AL was not described in *B. mori* (Oland and Oberlander, 1994b), extended their neurites remarkably in the culture with 20-HE without a change in the number of branches (Fig. 2). However, cultured AL neurons dissociated from stage-5 pupal brain of *M. sexta* respond only weakly, if at all, to 20-HE (Oland and Oberlander, 1994b) and this has also little direct effect on the morphological development of AL neurons (Oland and Hayashi, 1993).

Only two of the six neuronal types, which were identified in the AL of the *M. sexta* brain, responded to 20-HE (Oland and Oberlander, 1994a, 1994b). In one of those types, the effect was present only in neurons taken from the AL of the pupal brain at stage-5 prior to the arrival of sensory axons from the antenna and the effect was a decrease in the number of branches and in the total neurite length (Oland and Hayashi, 1993). In the other type, the effect was present, but only at the p<0.05 level of significance (Oland and Oberlander, 1994a, 1994b). In the culture with 20-HE in *B. mori*, therefore, only AL neurons of day-2 pupae, in which the AL has no ingrowth of sensory axons from the antenna (Kent *et al*., 1987; Oland and Hayashi, 1993; Oland and Oberlander, 1994b; Ai *et al*., 1998; Hansson and Anton, 2000), were used to exclude the possibility of direct effect of sensory axons on the morphological development of the AL neurons which we routinely followed.

Effect of 5-HT on neuronal growth

In the culture of all *B. mori* AL neurons with 5-HT, the effect of 5-HT were cell-type specific. Only the multi-polar neurons of four AL neuron types showed the specific morphological development in culture with 5-HT. Most of multipolar neurons were significantly stimulated to increase the number of branches projecting from the primary neurites.

The cell-type specific effect of 5-HT on the morphological development of AL neurons was also described in *M. sexta*. However, significant difference between effects of 5-HT on cultured AL neurons of *B. mori* and *M. sexta* is that in *B. mori* 5-HT stimulates only branching of the neurons, but in *M. sexta* it stimulates both neurite extension and the branching. Each of three AL neuronal types in *M. sexta* exhibited a type-specific response to 5-HT (Mercer *et al*., 1995). In particular, RR and FC neurons, which belong to the same major catagory of AL interneurons, were described to show type-specific responses to 5-HT (Oland and Hayashi, 1993; Oland Oberlander, 1994a, 1994b). RR neurons respond to 5-HT rapidly and the growth of neurons is enhanced by 5-HT for as long as it is applied. In contrast, the influence of 5-HT on FC neurons is limited to the promotion of the initial outgrowth of neurites from the cell body. Therefore, the effects of 5-HT on growth of cultured *M. sexta* AL neurons are both the extension of the neurite length in some neurons and the increase in the number of neurite branches in other neurons. In *B. mori*, however, 5-HT affects the AL multi-polar neurons only to increase the number of branches extended from primary neurites.

Effect of 20-HE on neuronal survival

Morphological and statistical data obtained from our experiments indicated that, on day-5, neurons of the day-2 AL, which could survive for up to 15 days in culture, showed significantly higher survival rates in a 0- or 0.2-μg/ml 20-HE titer than in a 1- or 10-μg/ml titer (p<0.05). There were no significant differences in survival between the 0- and 0.2-μg/ml doses. At day-7, AL neurons exhibited the highest survival rates in a 0-μg/ml 20-HE titer both with gradual decreases of survival rates in the order of 0.2-, 1- and 10-μg/ml titer and significant differences in survival rates at

each 20-HE titer.

However, there was no evidence for neuronal death in the metamorphic adult development during the rise in 20-HE titer from 0.2 to about 2 μg/ml that occurs between day 2–7 as in *M. sexta* (Sorenson, 1993). The discrepancy between the apparent absence of neuronal death *in vivo* and the increase in death *in vitro* on exposure to a higher 20-HE titer suggest that AL neurons give different weights to sometimes competing developmental cues (Oland and Hayashi, 1993).

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