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Indoleamine-accumulating Cell Death and Endogenous Glial Cell Reaction induced by 5,7-Dihydroxytryptamine in the Cat Retina

We investigated the patterns of degenerative changes of indoleamine-accumulating cells (IACs) induced by 5,7-dihydroxytryptamine (5,7-DHT, 100 μg), and the glial reaction to the neurodegenerative changes of IACs in the cat retina by using light-and electron-microscopy. The neurons accumulating 5,7-DHT in the cat retina were a few ganglion cells and displaced amacrine cells located in the ganglion cell layer (GCL), and some amacrine cells in the inner nuclear layer (INL). The cell density (per unit area, 1 mm^2) of the 5,7-DHT accumulating cells in the GCL and INL was 910 and 134 cells, respectively. Most 5,7-DHT accumulating cells showed dark degeneration characterized by widening of the cellular organelles at early stage, and by darkening of the cytoplasm at a late stage. In addition, amacrine cells, showing a typical filamentous degeneration, were observed in a few cases. The degenerated neurons were phagocytosed by microglial cells and astrocytes. The immunoreactivity for glial fibrillary acidic protein (GFAP) in Müller cells was increased at early stage, but thereafter abruptly decreased. In a few cases, severe degenerative changes were observed in Müller cells. These results indicate that 5,7-DHT induces severe dark degeneration of IACs, and most degenerated cells could be eliminated by microglial cells and astrocytes in the cat retina.

Key Words : *5,7-DHT, Indoleamine-accumulating cells, Degeneration, Glial reaction, Cat retina*

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INTRODUCTION

A population of indoleamine-accumulating neurons was first reported in the retina of the cat by Ehinger and Florén in 1976. A set of neurons that preferentially took up the 5-hydroxytryptamine (5-HT) analog, 5,6-dihydroxytryptamine (5,6-DHT), injected into the vitreous body of the mudpuppy, was identified by fluorescence microscopy (1). The specific uptake of indoleamines into mammalian retinae was later confirmed using either ^{3}H-5-HT uptake followed by autoradiography (2) or by immunocytochemical staining of 5-HT accumulating neurons (3-6). It is generally accepted that indoleamine-accumulating neurons comprise two distinct morphological types of amacrine cells and one class of ganglion cells in the cat retina.

5,7-dihydroxytryptamine (5,7-DHT) is a drug that is preferentially accumulated by the indoleamine-accumulating neurons in the central nervous system (CNS). It has been widely accepted that 5,7-DHT is toxic to the neurons in the CNS including the retina by free radicals (7-10). In the vertebrate retina, 5,7-DHT has been mainly used to identify the morphology and synaptic circuitry of indoleamine-accumulating neurons using its ability to degenerate neurons that have a high-affinity uptake system for 5,7-DHT (11-14). However, the detailed information on the degenerative processes of indoleamine-accumulating neurons induced by 5,7-DHT is still lacking in the mammalian retina.

In general, the pattern of neuronal cell death is subdivided into apoptosis (15,16), cytoplasmic degeneration (17, 18) and autophagic degeneration (19) during developmental period and under pathological conditions. Glial cells play an important role in eliminating the degenerated neurons and in reparing the lesion sites in CNS (19-22). A glial reaction is a common phenomenon in the CNS following the destruction of tissues, which may be induced by degenerative diseases or by trauma. Traumatic injury to the CNS results in gliosis at the site of the wound by proliferation or hypertrophy of the astroglial cells (20, 23, 24). The density of microglia is frequently increased in developmental or abnormal condi-

tions such as immunological changes and neurodegenerative dysfunctions (21, 22, 25, 26). In addition to the astrocytes and microglia, the mammalian retina contains a special type of glial cell, Müller cell, which normally gives the framework of the retina and plays a role in homeostasis. So it could be expected that the glial reactions in the retina might be different from those shown in other part of the CNS.

Therefore, the present study was carried out to clarify the patterns of the neuronal cell death and a glial reaction after administration of 5,7-DHT in the cat retina by light and electron microscopy.

MATERIALS AND METHODS

Animals

All experiments were performed using twenty adult cats of both sexes weighing 2.5 to 3.5 kg. They were housed in separate cages with a 12-hour light-dark cycle during experimental periods.

Treatment of 5,7-DHT

Cats were deeply anesthetized using sodium pentobarbital (30 mg/kg) via intravenous injection. They were intraperitoneally injected with 30 mg/kg body weight of dimethylimipramine (DMI, Sigma) and their pupils were bathed with 0.5% topical tropicamide. Before intravitreal injections, an anterior chamber paracentesis was performed. 100 μg 5,7-DHT (Sigma, USA) dissolved in 50 μl 0.9% NaCl were injected into the vitreous body using a 30-gauge needle, which was inserted 3 to 4 mm posterior to the limbus. In each animal, one eye was treated with 5,7-DHT, and the other one with 50 μl of 0.9% NaCl for the control.

Tissue preparation

Cats were sacrificed at intervals (1, 3, 7 and 14 days) after intravitreal injection of 5,7-DHT by an overdose of sodium pentobarbital, and the eyes were enucleated and hemisected. After removal of the vitreous body, the eyecup was immersion-fixed in a mixture of 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 24 hr at 4℃. The retina was separated from the pigment epithelium with a fine hair brush.

For light and electron microscopic observation, the tissue taken from superiotemporal region near the optic disc was dissected into samll pieces. They were postfixed in 1% OsO_4 for 2 hr at 4℃, dehydrated in a graded series of alcohol, infiltrated in acetone, and embedded in epon-araldite mixture. Semithin sections were stained with 1% toluidine blue for examination by light microscopy and ultrathin sections were stained with uranyl acetate-lead citrate. Ultrathin sections were placed on single-slot nickel grids coated formvar and examined in a transmission electron microscope (Zeiss EM 109, Germany).

Photoconversion procedure

To visualize IACs in cat retina, 100 μg 5,7-DHT was intravitreally injected and allowed to be stored by cells in the retina over 12 hr. Thereafter, the retina was gently isolated from the choroid using a fine hair brush. The wholemounted preparation was immersion-fixed in a mixture of 2.5% paraformaldehyde and 0.2% glutaraldehyde in PB for 2 hr at 4℃. After fixation it was rinsed twice for 10 mm in PB and photoconversion procedure was done.

A piece of the fixed tissue was rinsed with 0.1 M Tris buffer (TB, pH 8.2), and placed on a slide glass ganglion cell layer up. Excess TB was blotted off and the tissue was covered with a drop of diaminobenzidine solution (DAB 1.5 mg/ml in TB). Thereafter autofluorescent cells were viewed by conventional fluorescence microscopy. As the tissues were irradiated, the fluorescence in the cells faded and was replaced by an opaque reddish-brown DAB reaction product. During irradiation the DAB solution was rinsed off every 10 min and replaced with fresh DAB. The reaction was monitored under a light microscope and was stopped when the reaction product was sufficiently dense. The tissue was rinsed in TB and dehydrated, substituted and embedded for light- and electron-microscopy.

Immunohistochemistry

Some retinae were immersed in 30% sucrose in PB for 6 hrs at 4℃. They were rapidly frozen in liquid nitrogen and thawed at room temperature in order to enhance the antibody penetration. The retinal pieces were then embedded in 4% agar (Merck, Germany) and vertically sectioned with a Vibratome (PELCO 101, USA) at a thickness of 50 μm. The sections were transferred into 0.1 M phosphate buffered saline (PBS, pH 7.4) and processed for free-floating immunostaining.

Immunostaining was performed using the avidin-biotin-peroxidase complex (ABC) method. All sections were incubated in 10% normal goat serum (NGS) in PBS for 1 hr at room temperature and in a polyclonal antibody against GFAP (Sigma, USA) diluted 1:500 in PBS for 12 hr at 4℃. After being washed in PBS, sections were

incubated in biotinylated goat anti-rabbit IgG (Vector, USA) in PBS for 2 hr at room temperature and washed in PBS. They were then incubated for 2 hr with ABC solution at room temperature. After being washed in two changes of PBS and three changes of 0.05 M Tris-HCl buffer (TB, pH 7.4), they were incubated in 0.05% 3,3-diaminobenzidine (DAB) in TB containing 0.1% H_2O_2. The reaction was monitored under a light microscope and terminated by replacing the DAB and H_2O_2 solution with TB.

Specificity of immunostaining

One set of controls was run to test for antibody specificity. Normal rabbit serum (preimmune serum) was applied to the retinal tissues instead of primary antibody. No immunostaining was observed in control tissue.

RESULTS

Indoleamine accumulating cells (IACs) in the cat reina

To visualize the IACs in the cat retina, 5,7-DHT fluorescence was photoconverted in the presence of DAB under the fluorescence microscope. In wholemounted preparations, the relative position of the IACs could be identified by changing the focus of the microscope. Two populations of IACs could be clearly found at different depth of the focus within same fields of retinal wholemount. Many IACs both in the GCL and INL were labeled. The majority of the labeled cells (910 ± 175 cells/mm^2) were located in the ganglion cell layer (GCL), and some labeled cells (134 ± 65 cells/mm^2) in the inner nuclear layer (INL) (Fig. 1A). In addition, the IACs found in the GCL was heterogenous in terms of size of their somata and reaction intensity. The larger and densely stained cells (21.5 ± 3.7 μm) were more numerous, whereas smaller and lightly stained ones (9.2 ± 2.8 μm) very sparse. These cells seemed to belong to a class of ganglion cells and displaced amacrine cells, respectively (Fig. 1A, B). In the vertical section, a populations of IACs in INL could be readily observed. A small number of labeled cells, 8.7 ± 2.3 μm in mean diameter, were observed in the innermost part of the INL. These cells were thought to be conventional amacrine cells by their shape and position. The processes of IACs were also found to be diffusely distributed in the inner plexiform layer (IPL) (Fig. 1B, C).

Neurodegenerative changes of IACs

In the semithin sections stained with toluidine blue, general morphological changes of retinal tissues were clearly identified. Fig. 1D shows a vertical section of the normal retina with well-organized five layers; GCL, IPL, INL, outer plexiform layer (OPL), outer nuclear layer (ONL). At 1 day after injection of 5,7-DHT, no degenerative changes were observed. But at 3 days, cells in GCL taken up 5,7-DHT showed severe degenerative changes such as darkening of the cytoplasm, and pyknotic nucleus. The number of cells showing degenerative changes increased in the later experimental group (Fig. 1E). Some amacrine cells located in the INL also showed severe degenerative changes, such as shrinkage and darkness of the cell bodies, which were intercalated in normal amacrine cells. In addition, a few bipolar cells in the late experimental group (7 and 14 days) showed a typical dark degeneration. Under the light microscope, however, these cell were often confused with the degenerated Müller cells which are normally located between amacrine cells and bipolar cells in the mammalian retina.

In this study, the ultrastructural changes of IACs taken up 5,7-DHT were distinctly observed throughout the experimental periods. Morphological changes of IACs induced by 5,7-DHT in the cat retina were seen within 1 day of the injection. Swollen mitochodria appeared in the early experimental group, whereas various degenerative changes such as dilated cisternae of rough endoplasmic reticulum (rER), numerous vacuoles, and pyknotic nuclei appeared at late experimental group in the IACs located in the GCL. This finding demonstrates that mitochondria among cell organelles are much more succeptible to 5,7-DHT. Chromatolytic changes in the nucleus were occasionally observed in early stage of the experiment period. These pathological finding were continuously detected throughout the experiments. The cellular degeneration of IACs in GCL mainly occurred by necrosis. These necrotic cells showed a swollen appearence in their cytoplasm, and darkening changes with cellular shrinkage. These cells were found between intact ganglion cells not affected by 5,7-DHT (Fig. 2A).

The indoleamine accumulating amacrine cells also showed various degenerative changes by 5,7-DHT-induced cytotoxicity. The morphological alterations observed in these amacrine cells were quite similar to those in the ganglion cells of IACs. In the early stages of this experiment, a small number of amacrine cells showed a somewhat swollen appearance and contained a few disrupted cytoplasmic organelles. Subsequently, the amacrine cells exhibited severe cellular degeneraive signs, such as distorted mitochondria, increased number of lipofuscin granules and numerous vacuoles (Fig. 2B). As a result of the serial changes, the amacrine cells exhibited a typical dark degeneration already mentioned in the ganglion cell's death. But a different pattern of degenerative changes appeared in a few amacrine cells. These

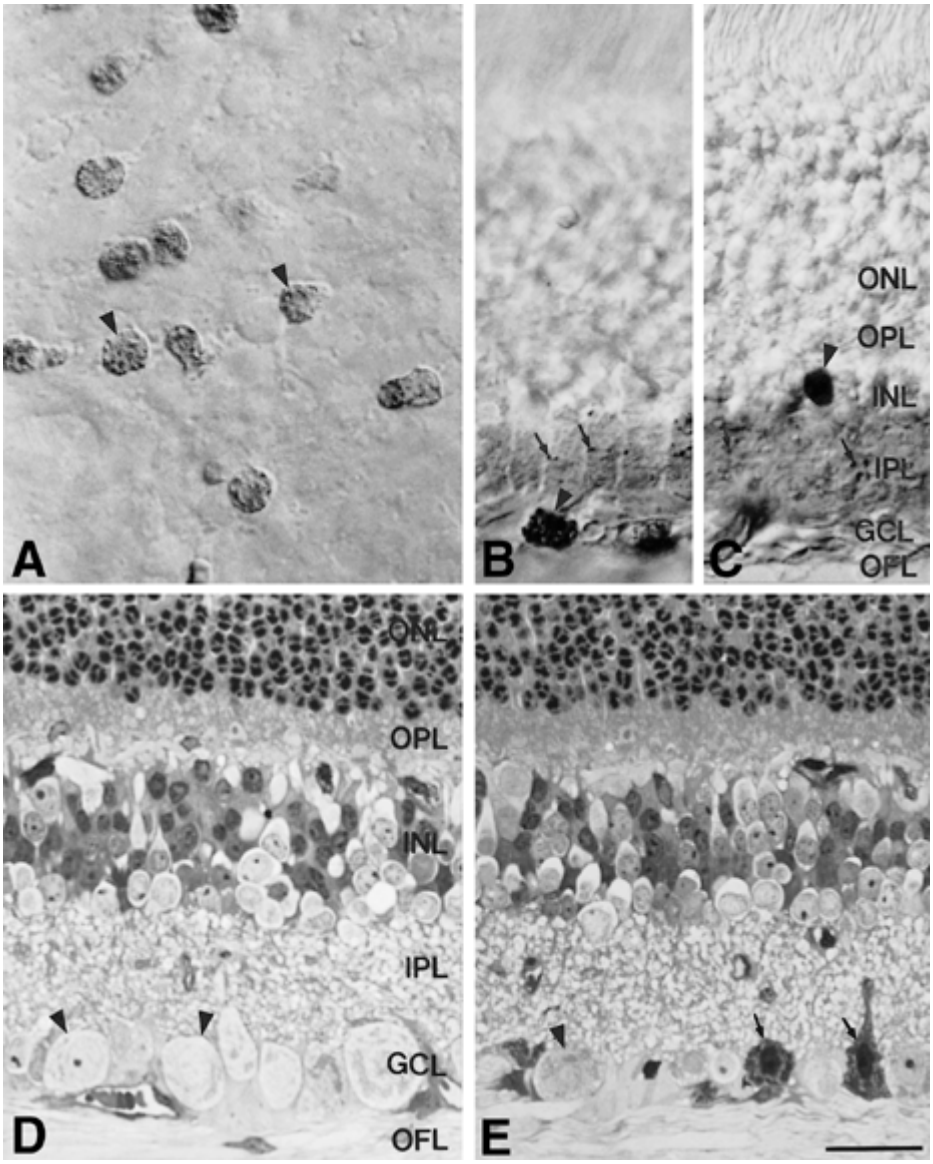

Fig. 1. Light micrographs taken from the whole mounted preparations (A) and vertical sections (B&C) of the cat retina accumulating 5,7-DHT, which was then photoconverted under a fluorescence microscope. Focused at the ganglion cell layer (GCL, A&B) and inner nuclear layer (INL, C), several cells (arrowheads) showing DAB reaction products are seen, respectively. 5,7-DHT accumulating processes are observed in the inner plexiform layer (IPL, arrows). Figs. D&E are light micrographs of the 1 μm thick vertical semithin sections of the normal retina (D) and retina 7 days after adminisration of 5,7-DHT (E). In D, a well-organized retinal structure is seen, and normal ganglion cells in the GCL are indicated by arrowheads. In E, a few degenerated ganglion cells (arrows) showing a severe degenerative changes such as shrinkage and pyknotic feature of the nucleus are noticed in the GCL. ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bar, 3 μm (D, E).

cells were filled with neurofilaments, showing a typical example of filamentous degeneration (Fig. 2C). In this study, a few bipolar cells were affected by 5,7-DHT induced cytotoxicity, and showed dark degenerative changes such as distented mitochondria, widening of rER including pyknosis. However, the occurrence and severity of the different ultrastructural alterations described above could not be sharply separated throughout the time course of this experiment.

Glial reaction to the neurodegenerative changes

In control cat retina, glial fibrillary acidic protein immunoreactivity (GFAP-IR) was mainly observed in the endfeet of Müller cells and in astrocytes, which were located in the GCL (Fig. 3A, D).

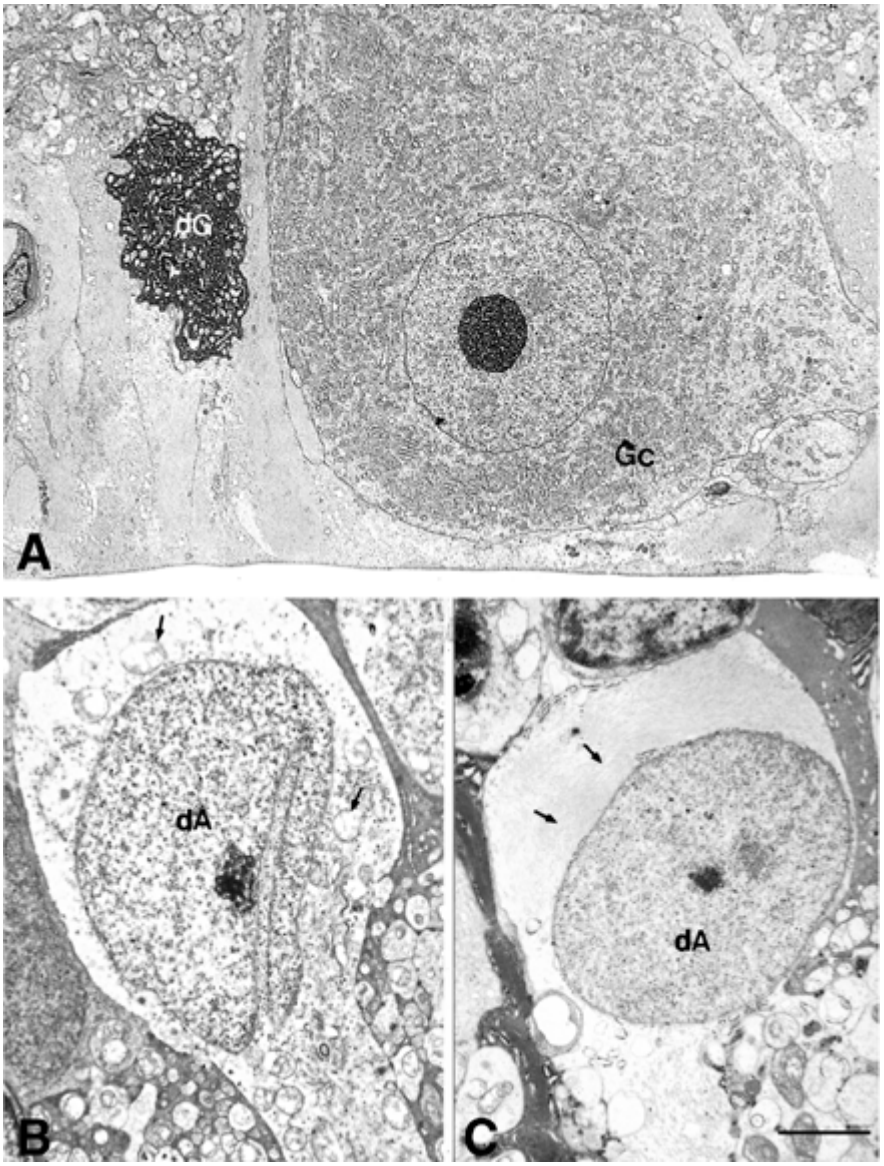

Fig. 2. Electron micrographs taken from a part of the ganglion cell layer (GCL) and inner nuclear layer (INL). A shows two different ganglion cells observed in 14 days after 5,7-DHT injection. On the left side of the photograph, a small ganglion cell (dG) undergoes severe degenerative changes, showing shrinkage and darkness of the cell body, but on the right side a large ganglion cell (Gc) shows normal appearence with well preserved organelles and has an oval nucleus with a prominent nucleolus. B&C are electron micrographs taken from a part of the inner nuclear layer at 7 and 14 days after 5,7-DHT administration, respectively. In B, an amacrine cell showing swelling of the cytoplasm with severe dissolution of the cell organelles, especially, mitochondria (arrows, dA). In C, an amacrine cell filled with a large amount of neurofilaments (arrows, dA) is seen. Scale bar, 3 μm.

In treated group, Müller cells showed GFAP-IR in response to intravitreal injection of 100 μg 5,7-DHT. At 1 day following the injection of 5,7-DHT these were confined to the IPL. At 3 days labeled radial processes became thicker and tortuous. They were observed to penetrate the ONL in a branching pattern. In addition, the labeled cell bodies were also found in the INL (Fig. 3B). At the electron microscopic level, the Müller cells also showed hypertrophic features such as abundant cytoplasm, and dilated cisternae of rER, suggesting that the activity of Müller cells was increased (Fig. 3E). At 7 days the cell bodies were more strongly labeled, but the labeled processes were somewhat decreased with compared to those of the earlier experimental group (Fig. 3C). By electron microscopy, Müller cells also showed mild degenerative changes, such as slight shringkage and higher electron density of the whole cells (Fig. 3F). At 14 days processes became very weak GFAP-IR and their length

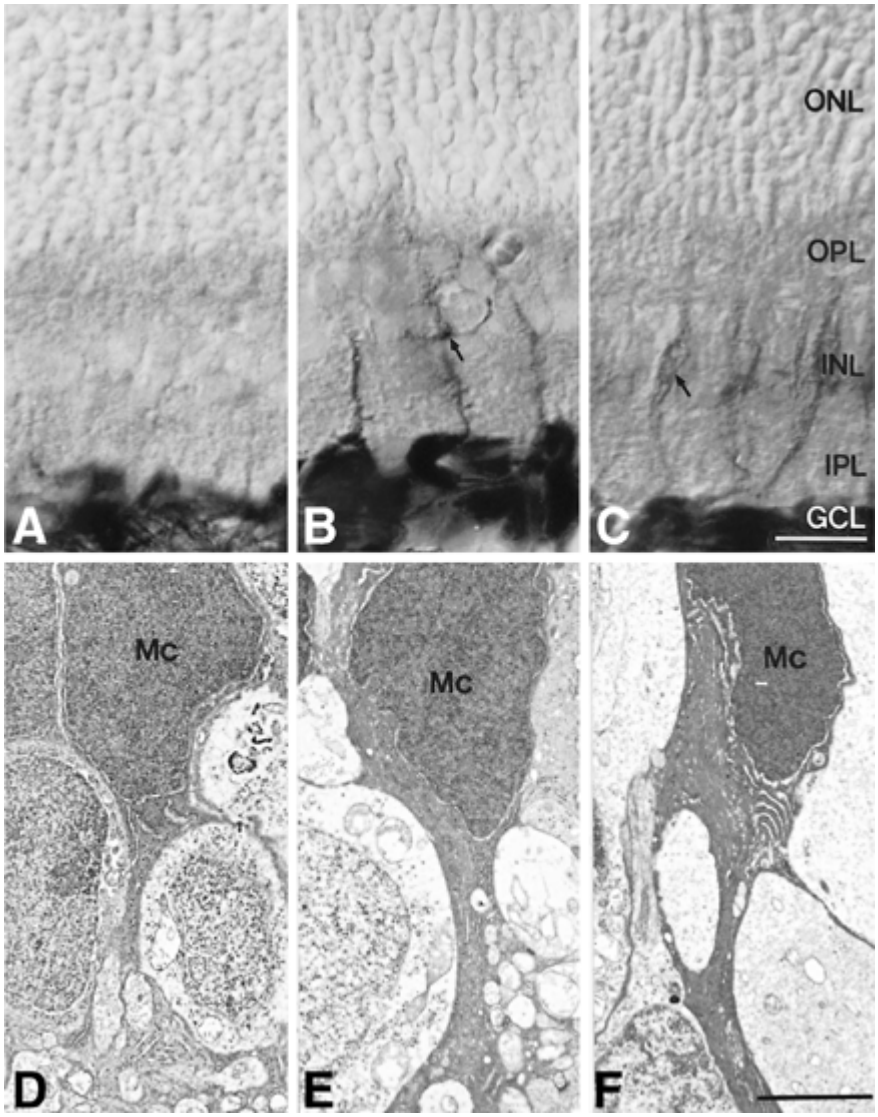

Fig. 3. 50-μm-thick vertical Vibratome sections of the normal retina (A), retinae of 3 (B) and (C) days after 5,7-DHT administration processed for GFAP-immunoreativity (IR). In A, GFAP-IR is confined to the cellular profiles located in the ganglion cell layer (GCL). In B, GFAP-IR is noticed in cell bodies of Müller cell (arrow) located in the inner nuclear layer (INL) and processes in the inner plexiform layer (IPL). In C, the pattern of GFAP-IR is similar to that shown in B, but the intensity is slightly decreased, and the cell bodies (arrow) are shrunken. Scale bar, 25 μm. Figs. D-F are electron micrographs of Müller cells taken from control retina (D) and retinae of 3 (E) and 7 days (F) after 5,7-DHT administration. In D, a Müller cell (Mc) with higher electron density are visible in the middle of the INL. Their scanty cytoplasm show poorly developed organelles. In E, a Müller cell (Mc) shows hypertrophic change such as widening of the cisternae of rough endoplasmic reticulum (rER). In F, a Müller cell (Mc) showing slightly degenerative changes such as narrowing of the cisternae of rER and increased electron density. ONL, onter muclear layer; OPL, outer plexiform layer. Scale bar, 2 μm (D, E, F).

became short, suggesting that there was no more distal processes to the INL. Therefore, GFAP-IR at 14days was very similar to that of the control. Under the electron microscope, some Müller cells were shrunken and darkened with disrupted cellular organelles, and thereafter most degenerating Müller cells continuously underwent dark degeneration of the cytoplasm.

No reactive microglial cells were found in the early stage, but the number of cells were considerably increased and their hypertrophic changes were frequently observed, especially, in the late experimental groups. These microglial cells acquired more abundant cytoplasm, so that they became larger in size and showed an irregular contour shape. At 7 days following the injection of 5,7-DHT, most microglial cells engaged in phagocytotic activity, and some degenerated cells engulfed by microglial cells were fre-

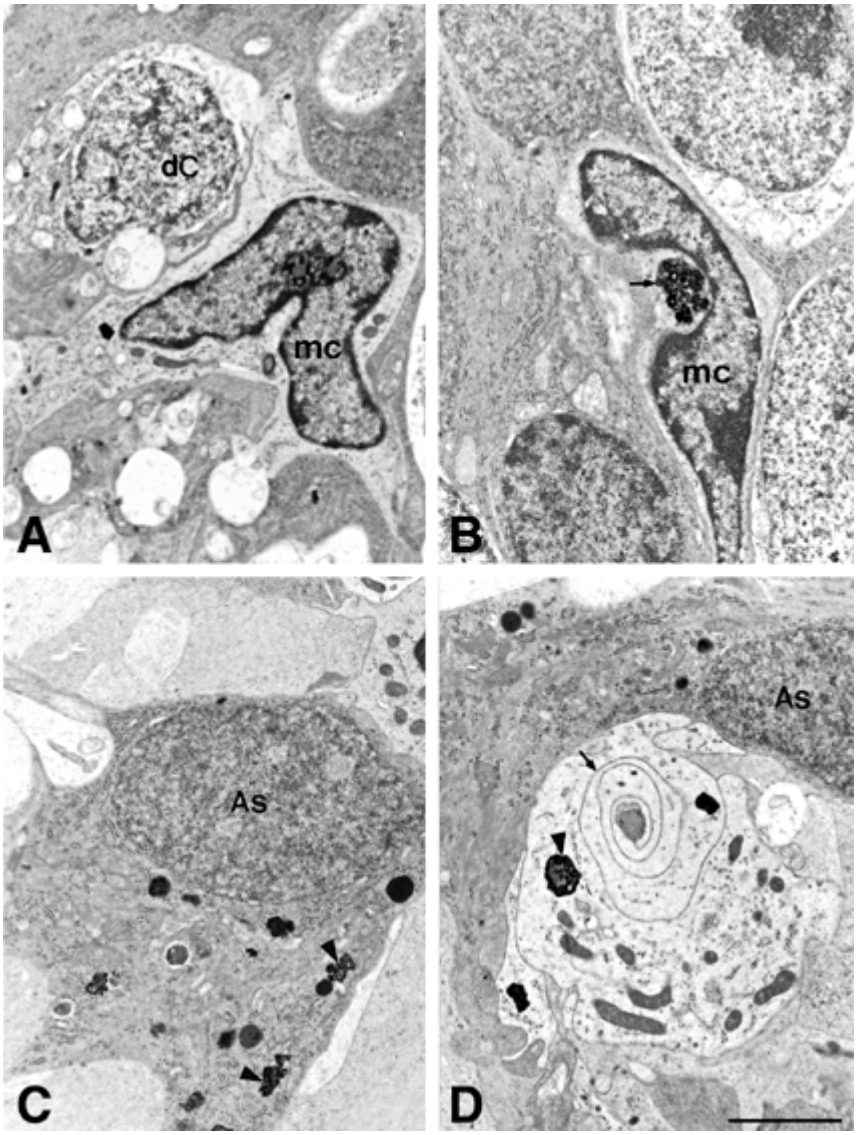

Fig. 4. Electron micrographs of microglia (mc) and astrocyte (As) taken from retinae 7 (A&C) and 14 days (B&D) after 5,7-DHT injection. In A, a microglial cell shows hypertrophic features with well developed organelles, and numerous cell porocesses directed toward degenerative amacrine cell (dC) in the INL. In B, and a phagocytosed debris (arrow) of a degenerated cell is engulfed by a microglial cell. In C, an astrocyte is seen to show hypertrophic changes such as abundant cytoplasm with well developed organells including lysosomes and cellular debris of degeneratd cells (arrowhead). In D, degenerated materials (arrowhead) are wrapped by a long slender process (arrow) of an astrocyte containing dense bodies in their cytoplasm. Scale bar, 2 μm.

quently observed (Fig. 4A). The phagocytotic nature of the microglia was characterized by the feature of degenerative products completely enclosed within the cytoplasmic processes at 7 day. At 14 days, cellular debris of the degenerated cells phagocytosed by microglia was observed in the INL (Fig. 4B) and GCL.

In the control group, most astrocytes were located either in the GCL or OFL. By electron microscopy, they were easily distinguished by their pale nuclei and abundant cytoplasm with bundles of glial filaments. During the experimental period, the prominent changes of GFAP-IR could not be found in astrocytes. At the electron microscopic level, hypertrophied astrocytes were observed, indicating that astrocytes are in active state (Fig. 4C). In addition, cellular debris of the degenerated neurons were occasionally wrapped by long-slender processes of the astrocyte, and the characteristic features were mainly observed in the GCL (Fig. 4D).

DISCUSSION

In this study, photoconversion provided a reliable method for the localization of fluorescent indoleamine markers in IACs. Most labelled cells by 100 μg 5,7-DHT are located in the GCL, and only a few in INL. Total numbers of labeled cell bodies in wholemounted preparation were about 910 cells/mm^2 in the GCL and 134 cells/mm^2 in the INL, respectively. IACs in GCL were seven times as numerous as IACs INL. This result contrasts with the previous report by Wässle et al. (6). According to their report, out of total IACs 77% were found in the INL and only 23% in the GCL. This discrepancy may be due to the dose and type of indected indoleamines. They injected 20 μg 5,6-DHT to identify serotoninergic neurons in the cat retina. In this study, higher dose was injected to induce degeneration of IACs. Taking into consideration with these results, ganglion cells in the cat retina might have dose-dependent uptake system of indoleamines.

The intravitreal injection of 100 μg 5,7-DHT produced toxic changes in the neurons accumulating the drug. In this study, most IACs showed degenerative changes such as swelling of cellular organelles, chromatolysis or pyknosis. These degenerative changes represent a typical form of the cytoplasmic degeneration. Such a degeneration has been reported in turtle (17), rat (21), rabbit (27), cat (28) and monkey (29) retinae which were administered by 5,6-dihydroxytryptamine (5,6-DHT). Cytoplasmic type of degeneration is thought to be common feature in degenerated neurons by indoleamines. In a few cases, amacrine cells which contained a large amount of neurofilaments were observed. This is a very interesting finding which has never been reported in mammalian retina investigated so far. This kind of degneration has been observed in lateral vestibular nucleus lesioned by axotomy in the rat (30), and termed as filamentous degeneration (30, 31) since neuron degenerate by accumulation of neurofilaments.

As previously mentioned, GFAP in an intermediate filaments protein, which is induced in the Müller cells by various pathological conditions (32-34). In this study, increased GFAP-IR emerged from 1 day after the injection of 5,7-DHT. The degree of expression became higher at 3 days after the injection. This result demonstrates that Müller cells can be active against neuronal death of IACs induced by the cytotoxicity of 5,7-DHT. In the early stage of this study, GFAP-IR appeared in the endfeet of Müller cell in the ganglion cell layer, but later GFAP-IR also appeared in Müller cell bodies. These results suggest GFAP in the Müller cells may be transformed from inactive from into active. From this point of view, Müller cells are considered to function similar to astrocytes in the brain. In the late stage of this experiment, however, GFAP-IR in the Müller cells became weak and similar to that seen in the control groups. At the same time, a few Müller cells exhibited severe degenerative changes, indicating that the Müller cells themselves suffered from the cytotoxicity of 5,7-DHT. However, further investigations are clearly needed to elucidate the cause of Müller cell death by 5,7-DHT.

In this study, reactive microglial cells appeared in the late stage, and they were engaged in the phagocytosis of cellular debris. This finding is in agreement with previous studies, which have reported that microglial cells are correlated with retinal cell death induced by neurotoxin (21) and optic nerve transection (25).

It has been well established that astrocytes produce neurofilaments to fill up for the defect area resulting from several pathological conditions (20, 24, 35, 36). In the present study, retinal astrocytes, in contrast to Müller cells, did not show prominent changes of GFAP-IR throughout the experiments. At the electron microscopic level, however, astrocytes with a number of lysosomes and wrapping degenerated neurons were observed. This finding strongly indicates that astrocytes also play a role as phagocytes in the pathologic condition of the retina.

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