DENSITY RATIO OF DOPAMINERGIC CELLS TO OTHER CELLS IN THE AMACRINE CELL LAYER OF THE FISH RETINA

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The retinas of mullet (*Mugil cephalus*) and carp (*Cyprinus carpio*) were processed for dopamine fluorescence, and were simultaneously counterstained with methyl green to obtain a differential nuclear fluorescence of other classes of cells. In tangential cryo-sections, the density was estimated for dopaminergic cells and other cells located at the innermost plane of the inner nuclear layer. In both the fish, the densities of the two classes of cells and the ratio of dopaminergic cells to other cells (mainly amacrine cells) were found to be higher in the peripheral region than in the rest of the retina.

Recently, dopamine-containing interplexiform cells have been found among amacrine cells at the innermost row of the inner nuclear layer (INL) of the *Cebus* monkey and teleost fish retinas (1, 2, 3, 5, 7, 18). The processes of these dopaminergic (DA-) cells have mutual synapses between amacrine cells in the inner plexiform layer, and they are presynaptic to horizontal and bipolar cells in the outer part of the INL (2, 3, 5). These cells thus provide a centrifugal (or feedback) pathway from the IPL to the outer plexiform layer (OPL). As an indicator of the physiological function of the DA-cells, pharmacological effects of exogenously applied dopamine have been explored on the membrane potential of bipolar and horizontal cells in the fish retina (2, 3, 14, 15, 16). It has been demonstrated that dopamine modulates the lateral spread of light-induced responses of horizontal cells (S-potentials).

On the other hand, the spatial distribution pattern of DA-cells has been studied with fluorescent flat-mounted preparations of the fish retina (17, 19). There has been one question as to what percentage of cells located in the innermost row of the INL are dopaminergic. It has been difficult to conserve dopaminergic fluorescence after or with counterstaining of other cellular elements. In previous studies of the fish retina (17, 19), the ratio of DA-cells among amacrine cells was estimated as 0.5% in flat-mounts of the carp retina, based on the amacrine cell density (10,000 cells/mm²) given by Tachibana (21) in tangential sections which were stained with toluidine blue. Recently, a simultaneous counterstaining technique of fluorescent preparations was developed using methyl green for the mouse submandibular glands by Nakamura *et al.* (12). We have applied methyl green to the carp retina in a further simplified manner, and succeeded in estimation of the ratio of dopaminergic

cells among cells located in the innermost row of the INL of the mullet and carp retinas.

MATERIALS AND METHODS

A histofluorescence (FGS) method, modified by Nakamura (11) from the Faglu method (6) was used to visualize dopaminergic (DA-) cells in the retina of mullet (*Mugil cephalus*) and carp (*Cyprinus carpio*). The solution for tissue fixation (FGS solution) consisted of 4% paraformaldehyde, 0.5% glutaraldehyde and 20% sucrose in 0.1 M phosphate buffer at pH 7.4. For counterstaining, 0.02% methyl green (MG) was added to the FGS solution.

The fish were dark-adapted for 1 hr before enucleation to facilitate the separation of the retina from the pigment epithelium. In order to accentuate the fluorescence of DA-cells, dopamine ($5\text{ }\mu\text{g}$ in $5\text{ }\mu\text{l}$ Ringer's solution) was injected intravitreally 2 hr prior to enucleation (17, 18, 19). Some of the isolated retinas were immersed overnight at 4°C in the FGS solution (routine fluorescent preparations). For the particular purpose of the present study, the retinas were fixed for 2 hr at 4°C in the MG-containing FGS solution, then transferred into the FGS solution (without MG), and kept overnight at 4°C . After fixation, the peripheral region of the retinas was cut apart from the central and intermediate regions, and both the retinal pieces were separately cryo-sectioned at -20°C radially or tangentially to $15\text{ }\mu\text{m}$ thickness. The three arbitrary concentric regions were determined by approximately equal divisions of the retinal radii, originating at the optic disc (17). The sections were dried over Drierite in a desiccator overnight at room temperature ($20\text{--}22^{\circ}\text{C}$), sealed with Entellan and examined under a fluorescence microscope (Nikon EF). The filter system consisted of a BG-3/IF-405 for excitation and a Y-475 for absorption.

RESULTS

Figure 1 illustrates radial sections (photomicrographs A, C and D) and a tangential section (B) of the mullet retinas (central regions), which were fixed in the FGS solution (A and B) and in the methyl green (MG-) containing FGS solution (C and D), respectively. One fluorescent cell seen in the center of photomicrograph A represents a typical shape of dopaminergic interplexiform cells (DA-cells) in the fish retina; processes emerging from the cell body extend towards both the outer and inner plexiform layers (OPL and IPL) and make fiber networks in the outer part of the inner nuclear layer (INL) and in the IPL, respectively. A tangential view of

FIG. 1. Fluorescence photomicrographs of dopaminergic cells of the mullet retina in radial (A and D) and tangential sections (B) of $15\text{ }\mu\text{m}$ thickness. A and B were obtained from preparations fixed in the FGS solution, whereas C and D were taken from the same radial section of sample fixed in a FGS solution containing methyl green (0.02%) for nuclear counterstaining. C was photographed under illumination of tungsten light, whereas D was taken under illumination of fluorescent light. The levels of the outer and inner plexiform layers (OPL and IPL, respectively) are indicated in A and D, and B represents the tangential section through the innermost level of the inner nuclear layer of such a preparation as in A. Two dopaminergic cells are marked by arrow with "d" (D). A-D $\times 300$.

a DA-cell is shown in Fig. 1B; 5 primary processes emerging from the cell body extend in various directions in the IPL. Photomicrographs 1C and 1D show the same area; C was obtained under illumination of tungsten light, whereas D was under illumination of fluorescent light. In color, the nuclei of all retinal cells stained with MG are deeply greenish in Fig. 1C, while these nuclei are reddish and the somata of DA-cells (marked by arrow with "d") are greenish in Fig. 1D as seen in Figs. 1A and 1B.

Fluorescence photomicrographs of tangential sections through the amacrine cell layer (the innermost cellular row of the INL) are shown in Fig. 2. They were obtained from the central or intermediate region (Figs. 2A and 2B) and from the peripheral region (Figs. 2C and 2D) of the carp retinas, which were fixed in the MG-containing FGS solution as was the sample in Fig. 1 (C and D). Figs. 2A and 2C were taken with lower magnification, whereas Figs. 2B and 2D were with higher magnification from corresponding areas, respectively. When comparing these pictures with Fig. 1B, the background of large DA-cell bodies (greenish in color) was fulfilled by small nuclei of other classes of cells (reddish in color). It can also be pointed out that the DA-cell bodies and the nuclei of other cells are larger in size on the average in the central and intermediate regions (Figs. 2A and 2B) than in the peripheral region (Figs. 2C and 2D).

Greenish fluorescent DA-cells and reddish fluorescent nuclei (assumably mainly of amacrine cells) were counted in each tangential section and expressed as the main density in the central and intermediate ("c + i") and peripheral ("p") regions, respectively. One photographic field (in color slide) covered a retinal area of 0.048 mm² (as in Figs. 2A and 2C). The results obtained are summarized in Table 1. It can be pointed out that the density of the two classes of cells and the

TABLE 1. *Densities (number/mm²) of dopaminergic (DA-) cells and other cells at the amacrine cell layer*

	"c+i"	"p"	"a"
A. <i>Mugil cephalus</i>	(n=10)	(n=16)	(n=26)
DA-cells	60 ± 4	134 ± 10	107 ± 8
Other cells	18,449 ± 1,008	25,185 ± 1,353	22,600 ± 1,047
%	0.33	0.53	0.47
B. <i>Cyprinus carpio</i>	(n=41)	(n=40)	(n=81)
DA-cells	40 ± 4	59 ± 3	50 ± 2
Other cells	16,796 ± 319	19,920 ± 252	17,720 ± 126
%	0.24	0.30	0.28

Numbers represent the mean ± 1 SEM. Numbers in parentheses are the numbers of photomicrographs examined, which were taken from 4 and 10 retinal samples of the mullet (A) and carp (B), respectively. The ratios of DA-cells/other cells are expressed as percentage (%). "c+i", retinal area includes the central and intermediate regions; "p", the peripheral region; "a", the average value from both the regions.

FIG. 2. Fluorescence photomicrographs of tangential sections through the amacrine cell layer, showing dopaminergic cell bodies and nuclei of other cells, counterstained with methyl green. A and B were obtained from the central or intermediate region of the retina, whereas C and D from the peripheral region. A and C × 300; B and D × 600.

ratio of DA-cells to other cells are higher in the peripheral region than in the central and intermediate regions for both the mullet and carp retinas.

DISCUSSION

Amacrine-like cells with processes extending towards both the outer (OPL) and inner plexiform layers (IPL) were commonly found in Golgi-preparations of many vertebrate retinas (1). On the other hand, histofluorescence studies revealed that the presence of dopamine-containing cells at the innermost level of the inner nuclear layer (INL) is a general feature of vertebrate retinas (1, 5). However, dopamine-containing cells with processes extending towards both the plexiform layers were found only in the retinas of *Cebus* monkey and various species of fish (2, 3, 5, 7, 17). This class of retinal cells was referred to as "dopaminergic interplexiform" cells (DA-cells in the present report), being differentiated from another set of dopaminergic amacrine cells in the vertebrates other than the *Cebus* monkey and fish species (1, 2, 3, 5). According to Boycott *et al.* (1), the interplexiform cells described from fluorescence microscopy may differ pharmacologically from those seen in Golgi-preparations.

The mean values of the DA-cell density listed in Table 1 for the mullet and carp retinas are comparable to those obtained from retinal flat-mounts of the respective fish (17, 19). The average value for the ratio of DA-cells to other cells is 0.28% (Table 1B) in the carp retina; this ratio is lower than the previously assumed value (0.5%), which was based on the amacrine cell density (10,000 cells/mm²) given by Tachibana (21). Since methyl green (MG) stains cellular nuclei, the cell number counted as the "other cells" includes mainly amacrine cells but also bipolar and Müller cells, located most proximally (21).

Histological studies (10) on goldfish revealed that as the eye grows the cell density decreases while the total number of retinal cells (except for rods) per retina increases. The DA-cell density in goldfish has also been found to decrease with retinal growth (13). Therefore, the comparison between the densities of DA-cells and of non-dopaminergic amacrine cells, obtained in different fish species or with different sizes in a given species, cannot be made easily. The body length (tip-to-tip) of the mullet and carp used in the present study was approximately 20 and 35 cm on the average, respectively. The mullet were at a rapidly growing stage, whereas the carp used were adult.

The present paper briefly described a new method for simultaneous fluorescent staining of DA-cells with other retinal cells in a methyl green-containing FGS fixative. The density ratio of DA-cells to other cells located in the amacrine cell layer was estimated as 0.47 and 0.28% in tangential sections of the mullet and carp retina, respectively. The density of both DA-cells and other cells, and the ratio of DA-cells to other cells were found to be higher in the peripheral than in the central and intermediate regions of the retina. According to Easter and his colleagues (4, 9, 10), the cell density of retinal cells (except for rods) decreases with retinal growth while the cell number per retina increases. Cell proliferation occurs only at the marginal zone neighboring the ora terminalis. Therefore, new retina are added so as to form rings. The rate of cell addition varies from fish to fish; the average rate is estimated as 12,000 cells per day. The reduced ratio of DA-cells to other cells in

the central and intermediate regions, found in the present study, may suggest that a small population of DA-cells in the peripheral region appears to become non-dopaminergic when the peripheral region of smaller retinas occupies the intermediate region of larger retinas, as increments of new retina are added at the margin.

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