MORPHOLOGICAL CHARACTERIZATION AND DISTRIBUTION OF INDOLEAMINE-ACCUMULATING CELLS IN THE RAT RETINA

Y. MATSUMOTO¹, S. UEDA² AND M. KAWATA²

*Department of Ophthalmology¹, Kyoto Prefectural University of Medicine and
Department of Anatomy², Kyoto Prefectural University of Medicine, Kyoto 602*

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Rat retina, which was intravitreally injected with 5-hydroxytryptamine (5-HT) or 5-hydroxytryptophan (5-HTP), was examined to identify indoleamine-accumulating cells by an immunohistochemical method in a flat-mount view and transverse sections. In the retina treated with 5-HT the immunostaining was observed in the cell processes, while in the retina treated with 5-HTP a large number of the immunoreactive cells with processes were noticeable. According to their morphological features and locations, they were divided into 5 types. Type 1 cells were large ovoid with a few processes and located in the inner lamina of the inner nuclear layer (INL). The majority of their processes ramified in the interface between the INL and inner plexiform layer (IPL). Type 2 and type 3 cells had large ovoid cell bodies, and the former was located in the ganglion cell layer (GCL) but the latter was placed in the IPL. Type 4 cells had small round cell bodies with a few fine processes and were placed in the inner lamina of the INL. Type 5 cells had the same features as type 4 cells but were displaced in the GCL.

These immunoreactive indoleamine-accumulating cells are categorized as amacrine cells from their shape and location. A morphological variety of indoleamine-accumulating cells in the rat retina indicates a wide range of involvement of amacrine cells in the signal transmission of visual information.

In the retinas of the vertebrates, the amacrine cells play a role in regulation and modification of the visual signals, which are transmitted through the photoreceptors, bipolar cells and ganglion cells to the brain (8, 16, 19). Recent histochemical studies (1, 2, 14, 15, 17, 18, 20) have shown various types of the amacrine cells on the basis of the neurochemical substances, and these cells show the different morphology and distribution in the retina. This morphological variety suggests that the amacrine cells have a wide range of different functions (7).

Serotonin (5-hydroxytryptamine, 5-HT) has been reported to be present in the retina of several vertebrates (9–11). The existence of the specific uptake systems for 5-HT or a closely related indoleamine in the vertebrate retinas suggested that indoleamines are taken up and released for neurotransmission. Ehinger and Florén (2) first reported the indoleamine-

accumulating neurons in the retinas of the rabbit, cat and goldfish with the use of Falck-Hillarp fluorescence histochemistry. Since then, these amacrine type of neurons which accumulate indoleamine have been demonstrated in the retina of the various vertebrates (3, 5, 12) by autoradiography and fluorescence histochemistry. In the rat retina, the presence of the indoleamine-accumulating amacrine cells was reported using the fluorescence histochemical methods (6), but there is as yet no strong evidence as to the existence of these neurons (3, 12). In the present study, we report the indoleamine-accumulating amacrine cells in the rat retina using immunohistochemical method in conjunction with an antiserum of 5-HT, and their morphological features and distributions.

MATERIALS AND METHODS

Tissue preparation

Twelve male Wistar rats, weighing about 300 g, were used in the present study. These rats were intraperitoneally given nialamide (a monoamine oxidase

Correspondence: Y. Matsumoto, Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602, Japan.

inhibitor) in a dose of 300 mg/kg. One hour later, five animals received bilaterally intravitreal injection of 5 μ l of 5-hydroxytryptamine (5-HT, 1 μ g/ μ l sterile saline), and 5 rats were given the injection 5 μ l of 5-hydroxytryptophane (5-HTP, a precursor of 5-HT, 1 μ g/ μ l sterile saline), and the other 2 rats were supplied the injection of 5 μ l of only sterile saline as control under ether anesthesia. Three hr after the injection, the animals were anesthetized with sodium pentobarbital, and perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4) and then a fixative (4% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, PB; pH 7.4). After both eyes of each animal were removed, an eye was stored in postfixative for one hr for flat-mount preparation. The eye was cut along the ora serrata, then vitreous bodies were completely removed and the whole retina was carefully extracted. The retina was given radially peripheral incisions in four directions (upper, lower, temporal and nasal). Only the upper incision reached the optic disk in order to make a topographical landmark. The retina was rinsed with 0.1 M PBS containing 1% Triton X-100 for several days at 4°C. The other eye was stored in the same fixative overnight and rinsed with 0.1 M PB containing 20% sucrose overnight. The eyeball was embedded into 10% gelatin. The hardened gelatin block was sectioned in the plane parallel to the meridian at the thickness of 25 μ m on a cryostat. The sections were rinsed in 0.1 M PB containing 0.3% Triton X-100.

Immunohistochemistry

The whole retinas and sections were treated with 1% normal goat serum overnight at 4°C, and then with rabbit anti-serotonin antiserum (working dilution 1:5,000) for 5 days at 4°C. They were further incubated with goat anti-rabbit IgG antiserum (Bio-Yeda, Israel, 1:200) for 3 hr and with rabbit peroxidase-antiperoxidase complex (Dakopatts, Denmark, 1:200) for 1.5 hr at room temperature. The whole retinas and sections were reacted with 20 mg of 3,3'-diaminobenzidine (DAB) in 100 ml of 0.05 M Tris-HCl buffer (pH 7.6) containing 20 μ l of 30% H₂O₂ for 10 min at room temperature. The whole retinas were flatly mounted on gelatin-coated glass slides and the sections were also mounted on the slides. Some of the sections were counterstained with cresyl violet. The preparation procedure and the specificity of the 5-HT antiserum were previously reported (13).

Microscopic analysis

Retinal flat-mounts and transverse sections were observed by light microscope. The retinal flat-mounts, in which immunoreactive cells were distinctly noticed, were evaluated for the determination of the

distribution analysis. Each quadrant separated by four incisions in the flat-mounted retinas was partitioned into a further 2 parts; the central and peripheral parts, at the half distance from the optic disk to the peripheral margin. Accordingly the retinal flat-mounts were divided into 8 parts; temporal upper periphery (TUp), temporal upper center (TUc), temporal lower periphery (TLp), temporal lower center (TLc), nasal upper periphery (NUp), nasal upper center (NUc), nasal lower periphery (NLp) and nasal lower center (NLc). The area of each part, the diameter of the cell bodies and the length of the processes were measured by the computer-based image analysis system (Histological tissue analysis system, Kashimura, Osaka, Japan). The immunoreactive cells were plotted using the camera lucida (Nikon, Tokyo, Japan) connected with the light microscope, and the number of these cells in each part was counted.

RESULTS

In the rat retina treated with only saline, no immunoreactive materials were found. In the retina supplied with 5-HTP numerous serotonin-immunoreactive cells (cell bodies and varicose processes) were observed (Fig. 1a), while in the retina treated with 5-HT the immunoreactivity was observed only in the fine varicose processes. The processes of these cells formed a network in flat-mount view. These immunoreactive cells were classified into 5 types according to the morphology and location.

Type 1 cells had large and ovoid shaped cell bodies ($18.8 \pm 2.5 \times 15.5 \pm 1.8$ μ m in mean diameter) with 2 to 4 primary processes (Fig. 1b). These cells showed the highest immunostaining among 5 types. The cell bodies were located in the inner lamina of the inner nuclear layer (INL), especially in its innermost lamina (Fig. 1c). The primary processes were thick and relatively smooth. The peripheral processes were rather thin and had various size of varicosities. Most of their processes lay in the boundary between the INL and inner plexiform layer (IPL). A small number of the processes extended in the middle lamina of the IPL (Fig. 1c). Occasionally, the processes proceeding outward across the INL to the outer plexiform layer (OPL) were observed (Fig. 1d). In the whole retina, the total number of type 1 cells was 771.8 ± 92.8 (mean number in five retinas) and the mean density was $27.2 \pm 2.6/\text{mm}^2$ (Table 1). The mean density of these cells in TUp was the highest among 8 parts. The distribution of type 1 cells was denser in the upper peripheral retina. The mean length of the processes

FIG. 1. 5-HT immunoreactivity in the retina. a. Low magnification of the flat-mounted retina. Bar=100 μ m b. Focused on the interface between the INL and IPL; Type 1 cell with 4 processes were observed in flat-mount view. Bar=20 μ m c. Type 1 cell remifies in the middle lamina of the IPL. Bar=50 μ m d. The process of type 1 cell reaches to the OPL. Bar=20 μ m OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer

TABLE 1. The density of type 1, 2 and 4 cells in 8 parts of retina

	TUp	TUc	TLp	TLc	NUp	NUc	NLp	NLc
Type 1	196.6 $\pm$ 22.5 (36.8 $\pm$ 5.1)	69.4 $\pm$ 16.3 (26.0 $\pm$ 5.7)	110.6 $\pm$ 31.6 (25.4 $\pm$ 6.7)	47.0 $\pm$ 11.0 (22.5 $\pm$ 4.1)	159.0 $\pm$ 27.0 (32.3 $\pm$ 2.5)	45.2 $\pm$ 4.8 (18.3 $\pm$ 1.3)	99.0 $\pm$ 30.1 (21.2 $\pm$ 5.9)	44.0 $\pm$ 6.9 (18.9 $\pm$ 3.8)
Type 2	8.6 $\pm$ 2.7 (1.6 $\pm$ 0.5)	0.3 $\pm$ 0.4 (0.1 $\pm$ 0.1)	5.6 $\pm$ 4.6 (1.3 $\pm$ 1.1)	0.3 $\pm$ 0.5 (0.2 $\pm$ 0.2)	8.0 $\pm$ 3.9 (1.7 $\pm$ 0.9)	0.3 $\pm$ 0.4 (0.1 $\pm$ 0.1)	1.0 $\pm$ 0.8 (0.2 $\pm$ 0.1)	0 (0)
Type 4	21.0 $\pm$ 5.2 (3.9 $\pm$ 1.0)	16.8 $\pm$ 4.5 (6.3 $\pm$ 1.8)	16.4 $\pm$ 8.3 (3.8 $\pm$ 2.0)	11.0 $\pm$ 2.9 (5.1 $\pm$ 1.4)	15.0 $\pm$ 3.2 (3.8 $\pm$ 1.4)	14.8 $\pm$ 3.6 (6.3 $\pm$ 1.8)	18.8 $\pm$ 0.9 (4.0 $\pm$ 0.4)	15.4 $\pm$ 2.5 (6.6 $\pm$ 0.4)

The total number and mean density (/mm², in parenthesis) of each type cell in 8 parts of retina are summarized. All data are the mean $\pm$ standard deviation obtained from 5 retinas.

TABLE 2. *The length of the processes and coverage of type 1 cells*

	TUp	TUc	TLp	TLc	NUp	NUc	NLp	NLc
the length of the dendrites	236±92	213±43	204±47	270±48	208±47	244±56	223±61	233±63
the number within the coverage	6.4±0.8	3.7±0.8	3.3±0.9	5.2±0.9	4.4±0.3	3.4±0.2	3.3±0.9	3.2±0.6

The mean length (μm) of the processes of type 1 cells is summarized and the number of the same type cells within the coverage of type 1 cell is calculated in each part of retina. All data are the mean $\pm$ standard deviation obtained from 5 retinas.

was $229 \pm 20 \mu\text{m}$ (Table 2). From calculation of these data, the mean number of these cells covered with the dendritic field was 4.1 ± 0.4 .

Type 2 cells were large ($18.3 \pm 2.1 \times 15.9 \pm 1.5 \mu\text{m}$ in diameter) and ovoid shape (Fig. 2a). They were displaced in the ganglion cell layer (GCL) (Figs. 2a, b). The arbores of the processes were intermingled with those of the type 1 cells in the interface between the INL and IPL. Two kinds of the proximal processes were distinguished: One is the process left from the outer surface of the cell body and directly ran outward (Fig. 2c), and the other traveled horizontally in a short distance and gradually proceeded outward. In the whole retina, the total number of this type cells was 23.5 ± 8.8 , and the mean density was $0.8 \pm 0.3/\text{mm}^2$ (Table 1). Most of these cells were distributed in the peripheral retina, especially in the upper peripheral part.

Type 3 cells were large ($18.4 \pm 3.0 \times 16.1 \pm 1.7 \mu\text{m}$ in diameter) and ovoid in shape, and the cell bodies were displaced in the IPL (Fig. 2d). The proximal processes ran horizontally within the IPL, and the peripheral processes extended in the same plane as those of the type 2 cells. The number of this type was very small.

The type 4 cells were small ($11.7 \pm 2.0 \times 9.5 \pm 1.6 \mu\text{m}$ in diameter) sized and of round shape (Fig. 2e). The majority of these cells possessed 2 or 3 fine processes and cells without having any processes were often observed in the flat-mount view. The cell bodies of this type were located in the inner lamina of the INL. The arbors of the processes lay in the same planes as those of the type 2 cells. In the whole retina, the total number of this type was 132.5 ± 16.4 , and the mean density was $4.6 \pm 0.6/\text{mm}^2$ (Table 1). The distribution density of these cells was higher in the central retina than in the peripheral retina.

Type 5 cells had small and round cell bodies ($11.5 \pm 2.3 \times 9.5 \pm 2.1 \mu\text{m}$ in diameter) similar to the type 4 cells, but they were located in the GCL. Their processes were hardly seen in the retinal flat-mounts, whereas the processes proceeding outward across the IPL were clearly shown in the transverse sections (Fig.

2f).

DISCUSSION

After Ehinger and Florén (2) first reported the presence of indoleamine-accumulating neurons in the retina of rabbit, cat and goldfish, these neurons have been histochemically shown in various species (3, 5, 12). In the rat retina, the indoleamine-accumulating neurons were demonstrated after the administration of 5,6-dihydroxytryptamine by using formaldehyde induced fluorescence histochemistry (6). However, there has been some contradictory evidence about the existence of these neurons in the rat retina (3, 12). In the present study of the rat retina in which rats were given 5-HT or 5-HTP, the indoleamine-accumulating cells with various morphological features and different locations were clearly identified by means of an immunohistochemical method in conjunction with specific antiserum of 5-HT. This discrepancy between the previous studies and our results may be due to the difference of sensitivity, because the former used fluorescence histochemistry and autoradiography to detect these neurons.

Our experiment of both the flat-mount and cryostat section of the retina of rats which were supplied with 5-HT showed the immunoreactive fine processes, while in the retina with the injection of 5-HTP high density of immunoreaction was observed in the cell bodies and processes. Since the antiserum used in the present study does not cross-react with 5-HTP, but with 5-HT (13), immunoreactive cells do contain 5-HT itself. This different localization of the immunoreactivities for 5-HT between the retinas given 5-HT and 5-HTP may be due to the difference of uptake, transportation and metabolism between these substances in the indoleamine-accumulating cells. Since 5-HTP is a precursor of 5-HT, it is probable that some amount of 5-HTP taken from terminals is transported to the cell body and converted to 5-HT in that place. However, at the moment it is out of our scope to conclude what the real mechanism occurs in terms of the relation between a precursor substance

FIGS. 2a-f. 5HT immunoreactivity in the retina. a. Focused on the GCL in flat-mount retina; the cell body (large arrowhead) and 2 proximal processes (small arrowheads) of the type 2 cell appear in focus, and the cell bodies (large arrow) of type 1 cells are shown out of focus. Bar=30 μ m b. Focused on the interface between the INL and IPL in flat-mount retina; the cell body (large arrowhead) and the proximal processes appear out of focus, and the peripheral processes (small arrows) and the type 1 cells (large arrows) are observed in focus. Bar=30 μ m c. The process of the type 2 cell runs vertically across the IPL in the transverse section. Bar=30 μ m d. The type 3 cell is located in the IPL. Bar=30 μ m e. Focused on the interface between the INL and IPL; the type 4 cell with a small round cell body (large arrowhead) and a process (small arrows) is seen. Bar=30 μ m f. The process of inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer

and transmitter in these cells. Further investigation is necessary.

The indoleamine-accumulating cells observed in the present study were divided into 5 populations. Large ovoid cells were located in the INL, GCL and IPL, whereas small round cells were placed in the INL and GCL. Most of the indoleamine-accumulating cells had a few processes. Judging from the criteria of its morphological features and locations (7), these cells could be amacrine cells. Sandell and Masland (12) demonstrated the morphology and distribution of the indoleamine-accumulating cells in the rabbit retina in rats which were given intraocular injection of 5,7-dihydroxytryptamine, and classified these amacrine cells into 5 groups. They suggested that the existence of five different types of amacrine cells reflect five pathways of phototransduction to a common effector site. Although the shape and distribution of indoleamine-accumulating cells observed in the present study are different in some aspects from those of rabbit retina, the variety of these cells in the rat retina indicates the involvement of amacrine cells in the regulation of the transmission of visual information through at least five different pathways.

A number of putative transmitter substances including GABA, glycine, dopamine, acetylcholine and a wide variety of neuropeptide-like substances such as cholecystokinin, enkephalin, glucagon, neurotensin, somatostatin, substance P, vasoactive intestinal polypeptide and corticotropin-releasing hormone are shown to localize to distinct populations of amacrine cells (4). The determination of the colocalization of these substances in the indoleamine-accumulating cells would be greatly important. Among these, dopamine is one of the strong candidates. Tyrosine hydroxylase-containing amacrine cells were identified in the rat retina using immunohistochemical methods (17). In this report, it was shown that 2 groups of amacrine cells are distinguished: the first group has a large ovoid cell body with a few processes and located in the INL and GCL, and the second group has a small round cell body placed in the INL and GCL. Moreover, this report demonstrated that the amacrine cells of the first group are populated in the upper retina with higher frequency than in the lower retina, and the density is from 20 to 30/mm². These morphological characteristics and distribution patterns of tyrosine hydroxylase-containing cells are quite similar to those of the indoleamine-accumulating cells observed in the present study. Further examination is necessary to determine whether tyrosine hydroxylase-containing cells have the uptake capacity of indoleamine.

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