Identification and Characterization of Tyrosine Hydroxylase Immunoreactive Amacrine Cells

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Using immunohistochemical techniques and antisera directed to the enzyme tyrosine hydroxylase, dopaminergic amacrine cells have been directly visualized in the rabbit retina. Tyrosine hydroxylase-like (TH) immunoreactive somata are observed only within the proximal inner nuclear layer (INL). These cells give rise to varicose processes, which are distributed to laminae 1, 3, and 5 of the inner plexiform layer. TH immunoreactive amacrine cells as observed from flat mounted, whole retina preparations have a medium- to large-sized somata. Their processes have extensive fields that provide at least a threefold coverage of any retinal region. As a population, these cells are located in all areas of the retina and are distributed in a sparse, nonrandom manner. These general features of the rabbit dopaminergic cell population appear to be representative of dopaminergic cell systems in other vertebrate retinas.


Dopamine is the primary catecholamine in the vertebrate retina.1 In rabbit, dopamine on the basis of chemical analysis is likely to be the only catecholamine present in amacrine cells.2,3 In this retina, as in others, dopamine uptake and release, the presence of dopaminergic synthetic and degradative enzymes, and high affinity dopamine receptor sites have been demonstrated.2,3 Morphological studies of rabbit retina have shown that dopamine is located within amacrine cells, which contribute their processes primarily, but not exclusively, to the outer portion (lamina 1) of the inner plexiform layer (IPL)4,5 where they make synaptic contacts.5 To date, the Golgi technique has allowed for a complete description of the morphology of individual neurons, but it has not allowed for an evaluation of the spatial organization of an identified retinal cell population, such as the dopaminergic cell population. With newly developed immunohistochemical techniques, it is possible to identify and characterize specific cell populations. Therefore, using these techniques with antisera directed to tyrosine hydroxylase, the rate limiting enzyme for the synthesis of dopamine, we have examined and begun a characterization of the dopaminergic cell system in the rabbit retina.

Materials and Methods

Antiserum

Antisera (#15A, #15B, #16) directed to tyrosine hydroxylase were generously supplied to us by Drs. A. W. Tank and N. Weiner of the University of Colorado.

Tissue Preparation

Adult, New Zealand albino rabbits obtained from commercial sources were used in these studies. The rabbits were deeply anesthetized with barbiturate and the eyes removed. The anterior segment was then cut away and the eye cup containing the retina was immediately immersed in 4% paraformaldehyde, 0.1 M D, L lysine HCl and 0.01 M sodium periodate in 0.1 M phosphate buffer (pH 7.2) for 1-2 hours at room temperature. Both sectioned and whole mounted retinas were prepared. If the retina was to be sectioned, the eyecup was then stored overnight in 30% sucrose in 0.1 M phosphate buffer with 10% normal goat serum and 0.5% Triton X-100. The retina was then processed...
according to a modified peroxidase-antiperoxidase immunohistochemical technique described below.

Immunohistochemical Procedures

Retinal sections were processed for immunohistochemical staining by either the immunofluorescence or peroxidase-antiperoxidase technique as described elsewhere. Briefly, the retinal sections were washed in 0.1 M phosphate buffer (with or without 10% normal goat serum) and then incubated for 12-48 hours at 4°C in antisera directed to tyrosine hydroxylase at a dilution of 1:1,000 with 0.3% Triton X-100 in 0.1 M phosphate buffer. The retinal sections were then washed in 0.1 M phosphate buffer, incubated in goat anti-rabbit IgG, conjugated to fluorescein isothiocyanate (Miles) at 1:100, washed in 0.1 M phosphate buffer, and finally coverslipped in a carbonate buffer-glycerin mixture.

For retina whole mount preparations, which were stained by a modified peroxidase-antiperoxidase (PAP) procedure, the retinas were incubated for 120 hours at 4°C in antisera directed to tyrosine hydroxylase at a dilution of 1:250 to 1:500 with 0.5% Triton X-100 in 0.1 M phosphate buffer. The retinas were then washed in 0.1 M phosphate buffer, incubated in goat anti-rabbit IgG at 1:20 (Miles) for 48 hours at 4°C, washed in 0.1 M phosphate buffer, incubated in peroxidase-antiperoxidase at 1:100 (DAKO) for 48 hours at 4°C, and washed in 0.1 M phosphate buffer. The retinal sections were then incubated with 3,3'-diaminobenzidine HCl (35-70 mg/100 ml) for 10 min after which 0.01% H2O2 was added to the incubation mixture and the sections were incubated an additional 5-10 min. Finally the retinas were washed, dehydrated and coverslipped. Specificity was assessed by substituting normal rabbit serum in place of the primary antiserum in all of these immunohistochemical procedures.

Results

Specific TH immunoreactivity is present within somata and varicose processes that are located in the proximal INL and IPL, respectively. In both sectioned and whole retina preparations, TH immunoreactive cells were observed in all retinal regions.

Retinal sections, taken perpendicular to the vitreal surface demonstrate that TH immunoreactive somata are located only in the proximal INL (Fig. 1). TH immunoreactive processes form a prominent, continuous plexus just below the INL in lamina 1 of the IPL. Other immunoreactive processes are sparsely distributed in more proximal regions of the IPL. They are mainly confined to laminae 3 and 5 of the IPL and are often discontinuous. The distribution of TH immunoreactive processes in laminae 3 and 5 of the IPL is more apparent in central retinal regions. TH immunoreactivity thus appears to be confined to amacrine cells which are only present only within the INL at the IPL border; no TH immunoreactive somata are observed in the ganglion cell layer (GCL). Moreover, there is no convincing evidence that TH immunoreactive processes are present in either the distal INL or the outer plexiform layer (OPL).

In flat-mounted, whole retina preparations, TH immunoreactive cells were clearly demonstrated within the INL (Fig. 2). They are characterized by medium to large somata and processes with extensive fields. The mean somal area for TH immunoreactive cells in peripheral retina is slightly larger than that for cells in the visual streak (Fig. 4B). TH immunoreactive somata are widely separated and usually give rise to two to four primary processes whose subsidiary branches extend for considerable distances. Processes from neighboring cells have considerable overlap, such that any given region of the retina lies within the fields of at least three different TH immunoreactive cells. These fields may even be greater, since the possibility that the finest processes of the TH immunoreactive cells are not seen in the whole mount preparations. This may be due to one or more reasons, including an absent or a very low concentration of tyrosine hydroxylase in these processes, the failure of the immunochemical reagents to penetrate these processes, and/or a failure to visualize very fine TH immunoreactive processes.

TH immunoreactive cell density varies across the retina (Fig. 3). For instance, retina #47, which is representative of other whole retina preparations, has a
Fig. 2. Photomicrograph of TH immunoreactive somata and processes in a whole mount preparation processed by a modified PAP technique. Photomicrograph is from the mid-periphery region of the inferior retina.

Fig. 3. Location of every TH immunoreactive somata in a flat-mounted rabbit retina prepared by a modified PAP technique. The retina was scanned with overlapping fields spaced at approximately 2.5 mm intervals with a viewing magnification of 31.25X. All stained somata in each field were plotted using a camera lucida, and the overlapping plots were combined to form the complete retinal map. The overlap was sufficiently large that each cell was plotted at least twice, and all discrepancies between overlapping plots were rechecked at higher magnification. This retina contained 8492 TH immunoreactive somata. The total retinal area measured planimetrically was 436.7 mm², giving an average density of 19.4 cells/mm². The visual streak, which lies just ventral to the myelinated fiber band and retinal blood vessels, had the highest density (about 23 cells/mm²) of TH immunoreactive cells. In superior and inferior peripheral retina, cell density is about 13 cells/mm². Finally, cell density is very low near the optic nerve head and beneath the myelinated fiber band. This reduced density is partly due to obscuration of the cells by retinal blood vessels.

An analysis of the distribution of TH immunoreactive cells was conducted on whole mounted retina preparations. TH immunoreactive cells were identified at random in the inferior retina, where cell density was relatively constant. For each cell selected, we measured the distance to its nearest neighbor and compiled these distances as relative frequency distributions (Fig. 4A). The smooth curves in Figure 4A illustrate the relative frequencies of distances to nearest neighbors that would be expected if the immunoreactive cells were randomly distributed (according to a Poisson probability rule) with densities equal to those observed experimentally. These theoretical curves do not match the experimentally observed relative frequency distributions. This observation suggests that TH immunoreactive somata are not randomly distributed. The somatic distribution of these cells was also analyzed using the statistical ratio, [mean/standard deviation of nearest neighbor distances]. Random patterns have a ratio of less than one. Higher values of this ratio indicate a higher degree of regularity. For the distributions of TH immunoreactive cells in Figure 4A, the ratios are 3.9 and 3.2 for retinas 54 and 47, respectively. These ratios suggest that TH immunoreactive cells form neither a random nor a highly regular pattern.

Discussion

This study demonstrates the presence of tyrosine hydroxylase-like immunoreactivity in a limited population of amacrine cells in the rabbit retina and confirms previous biochemical studies, which ascribe tyrosine hydroxylase enzymatic activity in the retina. Although earlier studies have described catecholamine fluorescence amacrine cells having a rather limited distribution of processes within the IPL, the present studies using more sensitive immunohistochemical protocols have demonstrated a more extensive distribution of processes. TH immunoreactive amacrine cells are similar in size and give rise to widely branching processes that are, for the most part, distributed to lamina 1 of the IPL. The present studies demonstrated that the dopaminergic cells give rise to extensive processes within the IPL and at least in central retina these processes are present in laminae 3 and 5 of the IPL.
cells are distributed throughout the retina and occur in higher density in the visual streak than in either the inferior or superior peripheral retina. TH immunoreactive cells in the rabbit retina can, thus, be generally described as being a wide field, sparsely branching amacrine cell type.

In rabbit retina, dopaminergic cells and their processes are found only in the proximal INL and IPL, suggesting that dopamine is not present in either interplexiform cells or displaced amacrine cells. The failure to detect either immunoreactive somata within the rabbit GCL and IPL or immunoreactive processes in the OPL in the sectioned and whole mounted retinas does not appear to be due to procedural difficulties. Since these identical immunohistochemical methods demonstrate TH immunoreactive displaced amacrine cells and interplexiform cells in the pigeon, cat, and monkey retina.

The present studies are consistent with other studies that indicate a low mean dopaminergic cell density in the rabbit retina. For instance, a density estimated of sectioned retinas treated by the Falck-Hillarp method reported 38 cells/mm². This estimate becomes 18 cells/mm² when it is recalculated using an Abercrombie correction for split-cell counts. This estimate, therefore, is similar to our estimate of dopaminergic amacrine cell density. A higher estimate of dopaminergic amacrine cell density has been reported on the basis of tritiated dopamine uptake autoradiography. It is not known if these higher values reflect either a sampling bias and/or nonspecific uptake of dopamine by some retinal cells. Regardless, all data indicates that the relative proportion of TH immunoreactive cells is extremely low. Although the total number of amacrine cells in rabbit retina is not known, a conservative estimate of 10⁶ amacrine cells in the INL suggests that the TH immunoreactive cells constitute less than 1% of the total amacrine cell population.

The density of dopaminergic cells in the rabbit retina is low in both absolute and relative number. This cell

Fig. 4. A, Nearest neighbor distance histograms for randomly identified TH immunoreactive cells in two retinas prepared as whole mounts using the modified PAP technique. The smooth curves plotted with each histogram show the expected relative frequencies of the somata were randomly distributed (the curves are given by \( f(r) = 2\pi r \exp(-\pi r^2) \), where \( r \) is nearest neighbor distance and \( \pi \) is mean cell density in cells/mm²). The poor fit between the experimental and theoretical distributions indicates that the TH immunoreactive somata are not randomly distributed. B Soma size distributions for TH immunoreactive cells in the visual streak and in peripheral retina. Mean soma area in the peripheral retina (134 µm²) is slightly but significantly larger than mean soma area in the visual streak (119 µm²; t-test, \( \alpha > 0.01 \)).
population is smaller than that reported for the glycine-, GABA-, and choline-accumulating amacrine cells.\textsuperscript{15-17} The sparsity of the dopaminergic amacrine cell population, however, is more than compensated by the extensively overlapping fields of their processes. The overlap of these processes is sufficiently large that, unlike the dendritic fields of ganglion cells in the cat retina,\textsuperscript{18} the fields of TH immunoreactive processes cannot be described by Dirichlet domains. Adherence to a Dirichlet domain model implies that the processes of neighboring cells interact strongly to establish mutually acceptable and largely exclusive territories. Thus it appears that the processes of dopaminergic cells interact only slightly or are formed independently of one another.

Even though the fields of neighboring TH immunoreactive cells may be independent of one another, their somal locations are not independent, as shown by their nonrandom distribution. This conclusion is supported by the observations that (1) the frequency distribution of TH immunoreactive cells does not match an expected random distribution and (2) the ratio of the mean/standard deviation of nearest neighbor distances indicates these immunoreactive cells are not regularly spaced. Interestingly, TH immunoreactive somata do have the same degree of regularity as $\alpha$-ganglion cells of the cat retina and displaced amacrine cells of the rabbit retina.\textsuperscript{7,10} Two possible explanations for the nonrandom somal distribution of TH immunoreactive cells are (1) that the cells are born with a nonrandom distribution that is maintained during retinal growth or (2) that a random pattern of dopaminergic cells at cell birth becomes more regular either by interactions between neighboring cells or by selective deletions (ie, cell death) as the retina matures. Whatever the mechanisms may be, the generation of some regularity of cell spacing may achieve an important simplification within the dopaminergic system, such that dendritic fields need only grow to a given size to achieve a fairly uniform coverage of the retina.

In summary, dopaminergic cells in the rabbit retina can be characterized as a population of low-density, nonrandomly distributed amacrine cells with extensive but sparsely branching processes. Our preliminary observations of TH immunoreactive cells in cat and pigeon retina,\textsuperscript{12} as well as studies of dopaminergic cells in fish and monkey retinas,\textsuperscript{13,19} suggest that, although there are differences in the exact laminar distribution of dopaminergic processes within the IPL, the spatial relationships of these dopaminergic cell populations are similar across these species.

**Key words:** dopaminergic amacrine cells, dopamine, tyrosine hydroxylase, immunohistochemistry, amacrine cell, rabbit

**Acknowledgments**

We thank Drs. A. W. Tank and N. Weiner for antisera directed to tyrosine hydroxylase, M. Cilluffo and L. A. Engsstrand for technical assistance, Drs. B. B. Boycott, W. K. Stell, and H. Wassle for helpful comments on an earlier version of this paper, and Drs. C. Gall and H. J. Karten for helpful comments on this version.

**References**