

Age-related Changes in Rat Retina

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Purpose: To describe the changes in rat retina occurring with ageing by means of histological methods, scanning electron microscopic observations and morphometrical data; and to study by means of biochemical methods the amount of protein content in retinal tissues.

Methods: Samples of fresh retinal tissue obtained from young, adult, and aged rats were studied by means of traditional histological methods and by scanning electron microscopy. Particular attention was paid to morphometrical data and to the changes which occur with ageing. With the aid of a quantitative analysis of images, a large amount of morphometrical data was collected. Moreover, the amount of protein content in retinal tissues has been determined.

Results: Retinal thickness significantly decreases with age. The ganglion cells seem to be more vulnerable to age-related loss than other retinal cells. The number of retinal capillaries is diminished with age. The intercellular connections between photoreceptors, the number of cellular processes, and the number of synaptic bodies of the bipolar cells also decrease significantly with age. These results were all confirmed by scanning electron microscopy observations and morphometrical findings. Biochemical dosage of proteins demonstrates that retinal tissues decrease with age.

Conclusions: All morphological, morphometrical, ultrastructural and biochemical data are concordant in demonstrating that the retinal tissues of rats undergo specific changes with age. Our findings are in agreement with those described by previous authors and underline that the rat retina can be considered an optimal model for studies on neuronal maturation and/or neuronal ageing. Since our data have confirmed that many changes occur in rat retina with ageing, we can hypothesize that rat retina is particularly sensitive to developmental changes and to senile decay. **Jpn J Ophthalmol 2001;45:68–75** © 2001 Japanese Ophthalmological Society

Key Words: Changes with age, quantitative analysis of images, retina, scanning electron microscopy.

Introduction

Age-dependent loss of nerve cells has been described in various layers of the rat retina.^{1–5} Retinal tissue possesses highly specialized neuronal cells with particular metabolic activity. The embryological origin of these cells is the diencephalon and the retinal cells therefore derive directly from the central nervous system.⁶ Increasing evidence seems to suggest that the decrease in visual acuity occurring in old age both in humans and in rats cannot be solely attributed to opacity of the cornea or of the lens but, at least in part, is dependent on the age-related changes at the level of the retina and/or of other portions of the optic pathways.⁷ Moreover, in the rat, the age-dependent loss of nerve cells in the retina does not seem to be a generalized phenomenon, but involves only some types of retinal structures.

Although the rat is widely used as a model for the study of neuronal ageing,⁸ to our knowledge quantitative data regarding age-dependent changes in some specific types of retinal structures (ie, capillar-

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ies, synaptic bodies, cellular processes, intercellular connections) are still lacking. On the basis of these considerations, the present study was performed to analyze whether these specific structures of the rat retina undergo quantitative age-related changes.

Materials and Methods

Retinas were obtained from 18 Sprague–Dawley male rats: 6 were 3 months old (considered as young), 6 were 12 months old (considered as adult) and 6 were 24 months old (considered as aged). The investigations were performed according to the guidelines of the Declaration of Helsinki. Animals were kept under standardized humidity, temperature, and light conditions (7 am-7 pm, light/dark cycle). They had free access to water and laboratory chow. The average lifespan of the rat colony used in our experiments was 24-26 months. The rats underwent intra-aortic perfusion with neutral-buffered paraformaldehyde after euthanasia by means of barbiturate overdose in conformity with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the American Veterinary Medical Association. After fixation in 10% neutral-buffered formalin for 2 hours, the retinas were trimmed to pieces of about 3 mm².

Pieces of retina from non-perfused animals were used for estimation of protein content. Pieces of retina obtained from perfused animals were used for morphological staining with toluidine blue and scanning electron microscopy. The thickness of rat retina differs in central, midperipheral, and peripheral areas, and for this reason the portions of retina used for our tests were always the midperipheral ones, near the macular zone. All morphological and ultrastructural data were submitted to a quantitative analysis of images. Finally, all morphological and biochemical results were submitted to statistical analysis of data.

Estimation of Protein Content

In all these experiments, samples of retina from non-perfused rats were weighed and homogenized into an ice-cold homogenization buffer (1/10 w/v). Tissue protein concentrations were determined using the method described by Lowry et al,⁹ using bovine serum albumin (BSA) as standard and Folin phenol as reagent.

Morphological Staining with Toluidine Blue

Pieces of the perfused rat retina were postfixed in 2% osmium tetroxide at pH 7.4 veronal-acetate

buffer for 1 hour at 4°C. After postfixation, the specimens were washed with veronal-acetate buffer (pH 7.4), dehydrated in a graded ethanol series and slowly air-dried. The post-fixed tissue was embedded in paraffin and ultrathin sections (about 1–4 μ m) were used for light microscopical analysis after morphological staining with toluidine blue (0.005% for 1 minute) to detect microanatomical details.

Scanning Electron Microscopy

Other pieces of the post-fixed tissue (treated in the same way as the ones used for morphological staining) were used for scanning electron microscopy. After fixation and dehydration, tissue samples were then oriented and the exposed surface was coated with gold-carbon vapor and examined in a JEM 100 B electron microscope with the EM Asid High Resolution Scanning Device (Pecs, Hungary). Photographs were taken using ORWO NR 5R NP 20 films. Microphotographs were printed in black and white.

Quantitative Analysis of Images

For a detailed evaluation of the effects of age on rat retinal morphology, a quantitative analysis was performed on slides and on microphotographs using the Quantimet Analyzer (Leica, Cambridge, UK) provided with specific software. This software enabled us to determine:

- 1. Thickness of the retina;
- 2. Number of ganglion cells/mm² of retina;
- 3. Number of capillaries/mm² of retina;
- 4. Number of synaptic bodies/observed area;
- 5. Number of cellular processes/observed area; and
- 6. Number of intercellular connections in the matrix between the photoreceptors.

All data were furnished directly by the display of the analyzer. The first three were expressed in cardinal units while the last three were expressed as conventional units (CU) \pm standard error of mean (SEM). These CU can be turned into numerical values by means of a transforming factor.

A converting factor may be applied to transform the CUs into cardinal units because by comparing the same values from the same images, in different experimental conditions, these values are representative from a comparative point of view. This method of quantitative analysis provides only data about the surfaces of the examined structures. If we also need to know the number of cells or capillaries we must use a converting factor as reported in the *Manual of Methods* for the equipment.¹⁰ A quantitative analysis of the ultrastructural details was performed on photographs by means of a Quantimet 500 Image Analyzer (Leica). Examinations were performed separately for each photograph evaluating the scanning electron microscopy.

The results obtained by this method may be incorrect because the main choices (the instructions for software) are decided by each research worker, according to personal preferences. For these reasons, the data may vary. It is necessary to follow the rules very carefully. The counts must be repeated at least three times using the double-masked technique. All the counts should be performed by different research workers, on different analyzers, and with samples identified only by a number or by a letter. Final results must be evaluated by another research worker, who examines experimental protocols to identify each sample and attribute specific values. Final values must be submitted to a statistical analysis of the data. The values reported in this paper represent the "values" of staining for each age group and are expressed as $CU \pm SEM$. The CUs are arbitrary units furnished and printed directly by the Quantimet system. Further details on this method of analysis are reported in the Manual of Methods for the Quantimet Leica 500 Image Analyzer.¹⁰

Statistical Analysis of Data

The statistical methods used throughout this study must be interpreted as an accurate description of the data rather than a statistical inference of such data. The preliminary studies of each value were performed with the aid of basic sample statistics. Mean values, maximum and minimum limits, variations, standard deviation, SEM, and correlation coefficients were applied as suggested by Serio.¹¹ Finally, a correlative analysis of the morphological and biochemical data was performed by comparing the significant differences for each age group with the corresponding values of other homogeneous groups. Correlation coefficients denote a significant level <.001 (P < .001) while the correlation coefficient is not significant when P > .05 (NS). The correlation coefficient was calculated according to Castino and Roletto.¹²

Results

All our results are described in Figures 1–8 and are summarized in Table 1. As can be seen in sections of the rat retina (Figure 1) stained with toluidine blue and with the same magnification $(50\times)$, all the retinal layers are recognizable. The pigment epithelium layer is detached from the outer segment of photoreceptors. By comparing Figures 1–c, we can observe a change in the thickness of the retina. The density of cells is also modified, as confirmed by quantitative analysis.

Microanatomical details of adult (12-month-old) rat retina, observed by scanning electron microscopy (magnification $4.800\times$), are reported in Figure 2. The outer segment of the photoreceptors, in contact

 Table 1. Values in Quantitative Analysis of Images of Morphological and Biochemical Changes in Rat Retinas at Different Ages

Morphological Changes*	Young $(n = 6)$	Adult $(n = 6)$	Old $(n = 6)$
1 Thickness of retina [†] ($\mu m \pm SEM$)	132 ± 21.4	210 ± 31.6	150 ± 18.3
2. Area of rat retina (mm^2)	31.1 ± 3.9	34.6 ± 4.1	19.2 ± 2.8
3. Ganglion cell area [‡] (mm ² ± SEM)	110826 ± 639	110418 ± 811	85836 ± 938
4. Capillary area (mm ² \pm SEM)	8.4 ± 1.4	7.3 ± 1.2	3.5 ± 1.2
5. Synaptic body/area (CU \pm SEM)	61.4 ± 9.5	62.6 ± 8.1	38.4 ± 6.6
6. Cellular processes/area (CU ± SEM)	44.2 ± 3.4	43.2 ± 2.8	28.1 ± 4.1
7. Intercellular connections/area (CU \pm SEM)	54.1 ± 7.2	52.2 ± 6.6	18.6 ± 3.5
8. Number of ganglion cell axons with			
diameter <0.4 μm	42136 ± 715	41328 ± 776	21622 ± 518
9. Number of ganglion cell axons with	31354 ± 819	30526 ± 229	29456 ± 232
diameter 0.4–0.6 µm	24296 ± 518	24312 ± 624	20721 ± 712
10. Number of ganglion cell axons with			
diameter 0.6–0.9 µm	1833 ± 21	766 ± 33	621 ± 27
11. Protein content γ /mg of fresh tissue	82.1 ± 1.5	76.3 ± 1.4	61.5 ± 1.4

*All values reported in 1–10 were obtained in same areas of young, adult, and aged rat retinas: 3–4 conversion factor enables us to change from area measurement to cell or capillary count. CU: conventional units in Quantimet system. SEM: standard error of the mean.

[†]Without detectable pigment epithelium in all layers.

[‡]Area occupied by ganglion cells corresponds to number of optic nerve fibers.



Figure 1. Sections of rat retina stained with toluidine blue 0.05%; (a) young (3 months old), (b) adult (12 months old), and (c) aged (24 months old). Note decrease in thickness of rat retina with age. Bar = $100 \,\mu$ m.

with the pigment epithelium layer, and the inner segment of photoreceptors showing numerous intercellular connections (arrows) are visible. The external limiting membrane represents the beginning of the inner and outer segments of the photoreceptors and separates them from the soma of the photoreceptors containing the cellular nuclei. Figure 2 was provided to show the rod outer segments in a 12-month-old rat. It would be advisable to also provide two figures showing the rod outer segments at the same site at the 3-month and 24-month-old stages in order to make the differences more apparent to the observer. The same considerations can be made for all the remaining figures. On the other hand, it is impossible to present a paper with 24 figures. For this reason we have performed the quantitative analysis on the figures at each stage. The values of the analysis make it possible for us to summarize a great number of results without showing a great number of figures. The outer segments of the rods of adult (12-month-old) rat retina, observed by scanning electron microscopy (magnification $14.400\times$), show the apex (a) of the segments covered by an intercellular matrix (Figure 3). Between the different segments the intercellular connections (ic) are visible. In many areas, the intercellular matrix appears to have been destroyed by experimental manipulations (arrow).

Outer segments of young (3-month-old) rat retina cones, observed by scanning electron microscopy (magnification 14.400×), clearly exhibit the apex (a) of the segments. The body of the segments is composed of "laminae" (L) coming from the cellular membrane and desquaming from the lower to the upper layers. The single segments communicate by means of intercellular connections (ic). The intercellular matrix has been removed by experimental manipulations (Figure 4).

The area between the bipolar and the multipolar cells of adult (12-month-old) rat retina observed by scanning electron microscopy is shown in the microphotograph Figure 5 (magnification $9.600\times$). The external nuclear layer made up of the so-called bipolar cells can be seen in the lower left corner. In the upper right corner the ganglion cell layer, formed by the so-called multipolar cells is clearly visible. Both these types of cells taken from the retinas of adult rats show a villous surface supplied by synaptic bodies (s) and cellular processes (cp). Numerous capillaries can be seen in the intercellular spaces. Figure 6 (magnification $9.600\times$) shows the same area as



Figure 2. Scanning electron microscopy of adult (12month-old) rat retina. OS: outer segment of photoreceptors, ic and/or arrows: intercellular connections, elm: external limiting membrane. Bar = $1 \mu m$.



Figure 3. Scanning electron microscopy of adult (12month-old) rat retina. Outer segment of rods. a: apex of segment, IC: intercellular connections, arrow: experimental artefact. Bar = $1 \mu m$.



Figure 4. Scanning electron microscopy of young (3month-old) rat retina. Outer segment of cones. a: apex of segment, IC: intercellular connections, L: desquaming laminae. Bar = $1 \mu m$.

shown in Figure 5 but as seen in the retina of an aged rat (24 months old). Both the ganglion and multipolar cells appear as large, spherical bodies, lying in a fibrous intercellular network. The vast majority of the cell bodies are smooth and do not present a villous surface. A presumed interplexiform cell shows a flat surface. An age-related decrease can be observed in capillaries (c), cellular processes (cp), and synaptic bodies (s), by comparing Figures 5 and 6 obtained from an adult and an aged rat, respectively.

Figure 7 presents the microanatomical details contained in a scanning electron microscopy micropho-



Figure 6. Scanning electron microscopy of aged (24-monthold) rat retina. IPC: interplexiform cell, CP: cellular processes, C: capillaries, S: synaptic bodies, Bc: bipolar cells, Mc: multipolar cells, gcl: ganglion cell layer. Bar = 1 μ m.

tograph of a retina taken from an adult (12-monthold) rat (magnification $4.800\times$). In the outer nuclear layer there is a presumed interplexiform cell, while other cells show numerous cellular processes and many synaptic bodies. Figure 8 is a microphotograph of the retina of an aged rat (24 months old, magnification $4.800\times$) in which the external limiting membrane, the inner segments of photoreceptors (is) and the external nuclear layer (enl) are evident. At this age the number of cellular processes and synaptic bodies present a marked decrease. All these morphological details were quantified and compared for



Figure 5. Scanning electron microscopy of adult (12month-old) rat retina. enl: external nuclear layer, gcl: ganglion cell layer, S: synaptic bodies, CP: cellular processes, C: capillaries, opl: outer plexiform layer. Bar = $1 \mu m$.



Figure 7. Scanning electron microscopy of adult (12month-old) rat retina showing cellular processes (CP), abundant synaptic bodies (S), and a presumed interplexiform cell (IPC). Bar = $1 \mu m$.



Figure 8. Scanning electron microscopy of aged (24month-old) rat retina. External limiting membrane (elm), inner segments of photoreceptor cells (IS), and external (outer) nuclear layer (enl) can be seen. Only one synaptic body (S) appears in close proximity to nucleus of photoreceptor cell. IPC: interplexi-form cell. Bar = 1 μ m.

each age by means of quantitative analysis. The related values are summarized in Table 1.

Examining the data reported in Table 1, some observations are possible: (1) The thickness of the rat retina is relatively small in young animals, becomes larger in adult ones and is significantly reduced in aged rats (about 60 μ m smaller than in adult rats; (2) the total extension of the retina is also strongly reduced in aged rats (about 20-25%) in comparison with young and adult animals whose total retinal area is similar; (3) the only values that seem to remain substantially unchanged in the three groups of animals are those that represent the total number of ganglion cells with axons having a diameter between 0.4 and 0.6 μ m, which seems to suggest that this morphological parameter is less susceptible to the ageing modifications, even though the total number of ganglion cells decreases by about 20-25% in aged rats in comparison to adult ones; (4) the data concerning retinal trophism as well as the area occupied by capillaries and the morphological characteristics of the retinal cells (ie, the number of synaptic bodies, cellular processes and intercellular connections/observed area) is quite similar in young and adult rats with non-significant modifications; however, these values appear to be strongly influenced by ageing since they are significantly reduced in the group composed of aged animals, suggesting that the main alterations are not only quantitative but also qualitative and functionally significant; (5) the lower number of axons with a diameter $<0.4 \mu m$ or between 0.6 and 0.9

 μ m, with respect to the axons whose diameter is 0.4–0.6 μ m which are substantially unchanged, seems to suggest a higher sensitivity to trophic alterations in small and large axons, compared with those with an intermediate diameter, as a consequence of the changes which occur with ageing; (6) the total protein content gradually decreases with ageing and this suggests a possible slow and progressive involution of the protein metabolism in the retinal tissue.

Discussion

In this study, particular attention was paid to morphometrical data and to the changes accompanying ageing in the rat retina. The thickness of the retina significantly decreases with age. The ganglion cells seem to be more vulnerable to age-related loss than other retinal cells. The number of retinal capillaries diminishes with age. The intercellular connections between photoreceptors, the number of cellular processes and the number of synaptic bodies of bipolar cells also decrease significantly with age. The scanning electron microscopy observations and morphometrical data confirm all these results. Biochemical dosage of proteins shows a gradual decrease in retinal tissues with age.

In the light of these results, some important aspects of retinal morphology can be analyzed: (1) ganglion cells; (2) interplexiform cells; (3) other scanning electron microscopy investigations.

Ganglion cells are quite evenly distributed in the rat retina, the variation between the highest and lowest density lies within a range varying from 6,000 to 1,000 cells/mm^{2.13} The total number of ganglion cells has been estimated to be 109,000–113,000.^{14,15} Perry et al¹⁴ divided retinal ganglion cells into three major types (I-II-III) on the basis of the axon diameter: (1) type I: only 1% of total cells with axonal diameter of $0.9 \ \mu m$; (2) type II: those cells with axonal diameter of 0.6 µm; (3) type III: those cells with axonal diameter of 0.4 µm. Ni and Dreher¹⁶ distinguished two additional subclasses within each of Perry's types II and III. The axons of retinal ganglion cells provide the unique output from the retina and the axons themselves are responsible for the constitution of the optic nerve. However, while the distribution of axonal diameters of the ganglion cells presents five peaks, the distribution of measured axonal diameters of the optic nerve shows only a single peak.^{17,18} Three groups of axons with different conduction velocities were, in any case, distinguishable by means of electrophysiological examination.¹⁹

Linberg and Fisher²⁰ focused their attention on the retinal outer plexiform layer in the human eye and stressed the role of the interplexiform cells and their specialized cell junctions. The latter junctions are similar to those existing between cones and flat bipolar cells and are in contact with the cone pedicles. Our results concerned only the interplexiform cells and their specialized cell junctions, ie, synaptic bodies. The external and internal plexiform layers of the rat retina possess synaptic bodies. These structures were first described by Polyak²¹ in 1941, then by Sjostrand²², and, more recently, by Hansson.²³ Other scanning electron microscopy investigations have made it possible to clarify the most characteristic features of retinal structure also by comparing the data obtained with that furnished by light microscopy.

Although scanning electron microscopy cannot reveal such fine details as transmission electron microscopy, it enables us to complete our knowledge about retinal organization. On the other hand, it may provide a far clearer demonstration of the sterical arrangement of the cells and intercellular structures than is possible using serial transmission electron microscopy studies, particularly for three-dimensional reconstruction purposes.23 Development of new scanning electron microscopy perspectives has made it possible to follow metabolic and dynamic changes in the tissues. Borwein and Hollenberg²⁴ studied the light- and dark-adapted states of teleost retina by scanning electron microscopy and transmission electron microscopy. They demonstrated clear differences between these two states, and found that the entire retina was wider when light-adapted and that, in particular, the rods were elongated. Cones, on the contrary, were longer in the dark-adapted state, and the inner-outer segment junctions were not so marked as in the light-adapted state. Nir and Ransom²⁵ also studied mouse photoreceptors during the dark/light cycle, with particular attention to intracellular distribution of arrestin in photoreceptor cells detected by immunocytochemistry and electron microscopy. Other interesting observations were reported by Krebs and Krebs²⁶ who analyzed the morphology of the primate retinal central fovea cells and correlated densities of horizontal and bipolar cells with the density of cone cell pedicles. Borwein et al²⁷ used scanning electron microscopy investigations to observe structural alterations after laser treatment of rabbit retina. Bird²⁸ and Meller and Telzlaff²⁹ studied the development of chicken retina, with particular attention to the formation of synaptic connection between the receptors and the bipolar cells. Fulton et al³⁰ studied the eyes of three patients with trisomy.¹⁸ Their transmission electron microscopy and scanning electron microscopy observations revealed cytologic details of typical immature neural retinas, as can be seen in developing chicken retinas.

Our findings corroborate those described by all the above authors and underline that the mammalian retina can be considered an optimal model for studies on neuronal maturation and/or neuronal ageing.

Since our data have demonstrated that many changes occur in the rat retina with ageing, we can hypothesize that the rat retina is particularly sensitive to developmental changes and to senile decay.

Our findings demonstrate that the rat retina undergoes specific changes with age. Our morphological, morphometrical and ultrastructural findings are concordant with the biochemical ones. In the retina of ageing rats, we can observe a decrease of thickness and capillaries, an only partial decrease in the number of ganglion cells, synaptic bodies and cellular processes and, finally, in the number of intercellular connections. Moreover, the reduction in the number of ganglion cell axons with a diameter <0.4 μ m or between 0.6 and 0.9 μ m, in comparison with the substantially unchanged number of axons with a diameter of 0.4-0.6 µm, seems to suggest that small and large axons possess a higher sensitivity to trophic alterations, compared with those of an intermediate diameter, as a consequence of the changes which occur with ageing.

The protein content in rat retinal tissues also decreases with age. For these reasons the rat retina may be widely adopted as a model for the study of neuronal ageing.

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