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No age-related cell loss in three retinal nuclear layers of the Long-Evans rat

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Abstract

The retina mainly contains ganglion, bipolar and photoreceptor cells which are distributed in the ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL), respectively. Whether there is an age-related loss of these retinal cells remains not well understood. Cell density and the total number of cells were two commonly used measures to evaluate such age-related changes in most previous studies and provided controversial conclusions. The use of density measures as decisive data is problematic because the total area of the retina was expanded in aging, whereas the application of the total number of cells was limited for assessing ganglion cells. In this study, thus, we wanted to test whether there is an age-related cell loss in the GCL, INL and ONL and if so, whether such a loss is correlated to the convergence ratio of these cells. We used stereological procedures to quantify the total number of cells in the three retinal nuclear layers in six young and six aged Long-Evans rats. We found that during aging, the total volume of the retina remained unchanged, but the retina became thinner. There was no cell loss in each individual nuclear layer, and the ratio of the ONL to INL to GCL was preserved.

Keywords: Cell number, Retina, Aging, Long-Evans rat, Stereological method

Introduction

Age-related brain cell loss is one of the significant alterations during senescence (Wickelgren, 1996; Peters et al., 1998; Rutten et al., 2003). Such alteration may extend to retinal cells that are derived from the embryonic diencephalon (Yuan & Yankner, 2000). Indeed, cell density in the rat retina (Cano et al., 1986; Katz & Robison, 1986; Weisse, 1995; Cavallotti et al., 2001), and human retina (Curcio & Allen, 1990; Gao & Hollyfield, 1992) significantly declines during life, being the primary basis for the above assumption. However, the use of density measures to evaluate age-related retinal cell loss is problematic as it assumes that the total volume or area of the sampled tissue has not altered during aging (Oorschot, 1994). Expansion and thinning of the retina during aging have been documented in the rat (Cano et al., 1986; Weisse, 1995; Harman et al., 2003). It was also found that the total number of retinal ganglion cells was unaffected by aging in the rat (Harman et al., 2003), quokka (Harman & Moore, 1999), rhesus monkey (Kim et al., 1996), and very few apoptotic cells were observed in the retina of the aging human (Dunaief et al., 2002). It thus remains to be further verified whether there is age-related cell loss in the retina.

The retina has various types of cells that are arranged in the ganglion cell, inner, and outer nuclear layers. Ganglion cells are the final output retinal neurons and collect the visual signal from photoreceptors via bipolar and amacrine cells. The convergence ratio of photoreceptors to bipolar cells to ganglion cells varies greatly, depending on the subtypes of these cells, and the species. For example, it was about 12 to 4 to 1 in the cone system (Kolb, 1979; Wässle et al., 1981*a*, 1981*b*), and 75000 to 5000 to 1 in the rod system (Sterling et al., 1988) in the cat retina. If there is an age-related loss of retinal cells, it has never been tested whether such a loss is proportionally corresponding to the convergence ratio of photoreceptors to bipolar cells to ganglion cells.

In this study, therefore, we used stereological procedures to quantify the total number of neurons in each nuclear layer of the retina in the young and aged Long-Evans rats. We found that during aging there was no cell loss in the retinal ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL), and the ratio of the ONL to INL to GCL cells in both young and aged retinae was roughly about 1:7:17. However, the retina became thinner in the aged rat, but the total volume of the retina remained unchanged.

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Materials and methods

Animals

The study had the approval of the Animal and Human Ethics Committee of Anhui Medical University. Twelve healthy Long-Evans rats were used in this study, including six young (6 months old, weighing 278 ± 45 g) and six aged rats (24 months old, weighing 732 ± 48 g), three males and three females for each age group. These animals were pigmented and have better binocular vision than those of albinos (Hupfeld & Hoffmann, 2006). The rats were raised under standardized barrier-breeding conditions (12-h light/12-h dark cycle) with free access to water and food. Each animal was anaesthetized with urethane and perfused with a saline solution followed by neutral-buffered formalin. The right eye was removed, postfixed, dehydrated, and then embedded in paraffin. A set of serial 5 μm sagittal sections was cut through the whole eye.

Sampling

A random and systematic sampling procedure for cell counting (Gundersen & Jensen, 1987) was performed at two stages: sampling sections and sampling counting frames (Fig. 1). Briefly, the sections were sampled at 375 μm intervals from each set of serial sagittal sections. The number of the sampled sections was 14.7 ± 0.8 in young rats and 17.0 ± 1.4 in aging rats. The sampled sections were stained with hematoxylin and eosin and examined using a Nikon 80i Microscope with a 100× objective. For cell counting, the counting frames were randomly and systematically sampled at an interval of five frames from the entire sampled sections. The number of the counted frames was 1544 ± 544 in young rats and 1370±492 in aging rats. The size of each counting frame was 10×10 μm^2 and cells within each counting frame were counted according to the forbidden-line rule (Gundersen et al., 1988*a*).

For measurement of thicknesses of the retina and each individual retinal layer, the measuring view fields were randomly and systematically sampled at an interval of five measuring view fields from the entire sampled sections. Each view field covered the entire thickness of the retina. The number of the measuring view fields was 77 ± 16 in young rats and 110 ± 23 in aging rats.

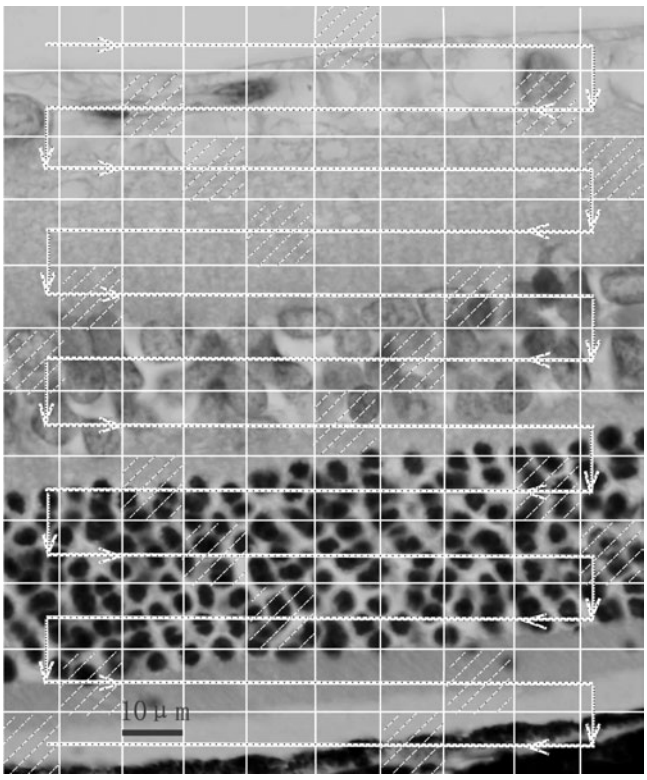

Fig. 1. Counting frames and counting method. The entire area of a sampled section was systematically scanned in a frame (10×10 μm^2) as indicated by the line with arrows. Counting frames (highlighted) were selected by choosing a random number ('6' in this example) from the random number table. Another random number ('6' in this example) was chosen to determine the first counting frame (at the middle of the first row). Cells within each counting frame were counted if the cells did not touch the left and bottom lines of the frame (the forbidden-line rule) (Gundersen et al., 1988*b*). The scale bar = 10 μm.

Estimation of the total number of retinal cells

The total number of cells in each retinal nuclear layer was obtained from multiplying the cell density of each layer by the total volume of the retina. The total volume of the retina was estimated by multiplying the average volume of the sampled sections by the total number of the sections per retina. The volume of each sampled section was determined by multiplying the volume of the counting frame ($10 \times 10 \times 5$ μm^3) by the total number of the frames that overlaid the entire section. The cell density of each nuclear layer was estimated with the formula: the total number of the counted cells divided by the total number of the counted frames and further divided by the volume of the frame.

Double counting problem

As 5 μm thick sections were used for cell counting, some nuclei may appear in adjacent sections, which will result in some cells being counted more than once. To circumvent this over-counting problem, we used the physical dissector method to examine 14 counting frames from adjacent sections of both young and aged rat retinae in order to estimate a correction factor for each retinal nuclear layer (Sanden et al., 2003; Oorschot, 1994). In brief, the nuclear profiles of the counting frame from two adjacent sections were traced onto a clear plastic sheet, respectively. The plastic sheets were then superimposed on one another so that the profiles could be matched to assess which individual nuclear profiles were present in both sections. The correction factor was determined by dividing the total number of the nuclear profiles that were not present in the previous section by the total number of the nuclear profiles in the section.

Statistical analysis

The data were analyzed using the one-way ANOVA to detect differences between the age groups. Differences were considered significant when $p < 0.05$. No statistical difference was found between sex groups (data not shown).

Results

Thickness and volume of the retina

On a hematoxylin and eosin stained section, the retina displays the nerve fiber layer (NFL), GCL, inner plexiform layer (IPL), INL,

outer plexiform layer (OPL), ONL, photoreceptors (PR), and retinal pigment epithelium (RPE). We measured the thickness of the whole retina as well as that of each individual layer in both young and aged rats. The average thickness of the aged retina was 109 μm, which was 36% thinner than that of the young retina (Table 1). All of the individual retinal layers were significantly thinner in the aged rat, with the exception of the NFL where no change was found between the age groups (Table 1). However, no difference in the total volume of the retina was found between the age groups ($p = 0.496$) (Table 2). Taken together, the results indicate that the average area of the aged retina is enlarged relative to the young retina.

Total number of retinal cells

Table 2 summarizes the total number of retinal cells in the two age groups. No significant differences were found between the groups ($p > 0.05$). Furthermore, although the aged retina was thinner than the young retina (Fig. 1 and Table 1), there was no significant difference of the cell density (per mm^3) in the three nuclear layers (GCL, INL, and ONL) between the two age groups (Table 2). Based on the total number of the nuclei in the GCL, INL, and ONL (Table 2), we estimated that the ratio of the ONL to INL to GCL cells was roughly about 17 to 7 to 1 in the both young and elderly retinae.

Discussion

In this study, we used stereological procedures to quantify the total number of cells in the three cellular layers of the young and aged rat retina in order to test whether there is an age-related cell loss in the three retinal nuclear layers, and if so, whether such a loss is correlated to the convergence ratio of the ONL to INL to GCL cells. The results demonstrate that during aging there is no appreciable loss in any layer, and the ratio of the ONL to INL to GCL cells remains unchanged. The retina becomes thinner, but total retinal volume is preserved.

Whether there is an age-related cell loss in the retina is still controversial. Our results are consistent with those studies in which the total number of retinal ganglion cells was estimated (Kim et al., 1996; Harman & Moore, 1999; Harman et al., 2003) but against other studies in which the cell density (per unit area) was used for comparisons between age groups (Cano et al., 1986; Gao & Hollyfield, 1992; Weisse, 1995; Cavallotti et al., 2001). The use of density measures as decisive data is problematic as it is based on the assumption that the total volume or area of the sampled tissue has not altered (Pakkenberg et al., 1991; Oorschot, 1994). Evidence from this study (Table 1) and previous studies (Cano et al., 1986; Weisse, 1995; Cavallotti et al., 2001; Harman et al., 2003) proved that the thickness of the retina significantly decreased whereas the area of the entire retina increased during the aging process. Such alterations inevitably affect the estimation of the cell density in a given region of the retina because the volume of the region has changed whereas the cell density is determined by two factors: cell numbers and volume. Thus, quantification of the total or absolute number of retinal cells provides much more reliable data to assess the age-related cell loss. Furthermore, the previous studies assessing the total number of cells in the retina were limited in the GCL as the cells in this layer are normally distributed as a single cellular layer that can be visualized on a whole mounted retina (Kim et al., 1996; Harman & Moore, 1999; Harman et al., 2003). However, the whole mounted retina is not suitable for assessing the total number of cells in the INL and ONL where the multiple-layered cells overlap each other (Fig. 1). The stereological method used in this study overcomes the above problem and allows us to estimate the total number of the cells in each individual layer of the retina, from which we can calculate the ratio among them.

Recent studies with the application of state-of-the-art stereological methods have provided convincing evidence that age-related loss of cells in the brain is associated with specific regions of the brain and specific types of neurons (Rapp et al., 1996; Long et al., 1999; Rutten et al., 2003). Data provided in this study indicate that the number of retinal cells is not affected by aging. However, the conclusion of no change of the total number of the retinal cells during aging does not rule out the possibility of a regional retinal cell loss because the retinal cells are not evenly distributed, e.g., no retinal cell at the optic disc and the photoreceptor cells predominating at the foveola and macula. Some specific regions of the retina may have a substantial age-related loss of cell (Curcio et al., 1993) but the number of such lost cells may be too small to be detected when compared to the total number of the whole retinal cells. Indeed, it is difficult to verify

Table 1. *Comparison of the thickness (μm) of the retina between young and aged Long-Evans rats**

	Young ($n = 6$)	Aged ($n = 6$)	P value
Retinal thickness	170.5 ± 28.0	108.5 ± 37.1	0.003
Individual layers			
NFL	8.3 ± 1.1	6.5 ± 2.2	0.100
GCL	10.6 ± 1.5	8.1 ± 2.4	0.000
IPL	43.1 ± 8.2	26.5 ± 13.1	0.000
INL	26.5 ± 7.6	15.3 ± 7.5	0.000
OPL	11.5 ± 2.1	6.2 ± 2.7	0.004
ONL	38.9 ± 5.7	24.4 ± 9.2	0.008
PR	24.0 ± 5.8	15.2 ± 2.8	0.008
RPE	8.3 ± 1.2	6.5 ± 1.6	0.049

*The values shown in the table are the mean values ± standard deviation.

Table 2. *Comparison of the total number of retinal cells in three nuclear layers between young and aged long-evans rats**

	Young ($n = 6$)	Aged ($n = 6$)	p value
Total volume of retina (mm^3)	2.12 ± 0.70	1.85 ± 0.65	0.496
Cell density per retina ($\times 10^6$/mm^3)			
GCL	0.13 ± 0.04	0.17 ± 0.06	0.303
INL	0.95 ± 0.31	1.03 ± 0.19	0.849
ONL	2.26 ± 0.71	2.66 ± 0.55	0.299
Total number of cells per retina ($\times 10^5$)			
GCL	2.12 ± 0.29	2.27 ± 0.48	0.522
INL	15.53 ± 2.28	15.55 ± 4.12	0.993
ONL	39.46 ± 99.30	41.50 ± 10.72	0.739

*The values shown in the table are the mean values ± standard deviation. Total number of cells per retina has been corrected for double-counting. The correction factors for the GCL, INL, and ONL were 0.18, 0.15, and 0.11, respectively. No significant difference was found between the age groups ($p > 0.05$).

such a regional cell loss in the retina using the measure of the total or absolute cell number because the boundary of those regions may not be precisely defined.

The present study has at least three limitations. First, the use of 5 μm thin sections may overestimate the total number of retinal cells because of the double counting problem (see Material and method section for details). To circumvent this over-counting problem, we used the physical dissector method to examine 14 counting frames from adjacent sections of both young and aged rat retinae in order to obtain the correction factor for each nuclear layer. The obtained correction factor was then applied to correct the estimation of the total number of cells in each nuclear layer, which was based on the single-section examination (Table 2). Thus, the total number of cells presented in Table 2 is an indirect estimation. It is better to use either the physical dissector, in which the adjacent sections are used, or the optical dissector, in which the single thick sections (e.g., thickness of 40 or 60 μm) are optically sectioned, to directly estimate the cell number in each nuclear layer (Oorschot, 1994).

Second, this study examined the retinal cells rather than the retinal neurons because in the hematoxylin-and-eosin-stained-sections, the retinal neuronal and non-neuronal cells were unable to be discriminated. Thus, care should be taken when interpreting our results. For instance, the total number of the retinal cells in the GCL (Table 2) should not be compared with the total number of the ganglion cells reported in previous studies (Perry et al., 1983; Harman et al., 2003; Cavallotti et al., 2003; Cepurna et al., 2005) because the nuclear profiles counted in our study included not only the ganglion cells but also other supporting cells, e.g. amacrine cells, which may make up 50% of the neurons in this layer (Perry, 1983). Thus, our conclusion that during aging there is no appreciable cell loss in the GCL would not exclude the unlikely possibility that ganglion cell numbers decline while supporting cell numbers increase during aging, which results in an unchanged overall numbers of cells in the GCL.

Third, this study only estimated the total volume of the whole retina and did not attempt to include the volume of each individual retinal layer. Changes of the volume may vary between different retinal layers. For instance, extents of the thinning of individual retinal layers were different (Table 1). While the thickness of the majority of the retinal layers decreased over 36%, there was no change in the NFL (Table 1). Thus, the volume of the NF layer may actually increase as the whole retinal area is expanded whereas that of the other layers may be stable or decrease during ageing.

In conclusion, most previous studies suggest that age-related retinal cell loss is a common feature during the retinal aging process. We report here for the first time that the total number of retinal cells during aging remains unchanged, but the thickness of the retina is significantly reduced.

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