Age-Related Alterations of Proteins in Albino Wistar Rat Retina

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Keywords
Immunohistochemistry · Western blot · Morphometric analysis · Retinal layers · Histology

Abstract
Imbalance of homeostasis causes permanent changes in the body with time. The central nervous system is especially prone to these changes since it possesses limited regenerative capacity. In the retina, neurons are damaged during the aging process, and this eventually leads to deterioration of vision. In our 2-year-long study, we examined genetically closely related rat individuals to disclose the hidden retinal causes of age-associated visual dysfunction. Morphometric analysis showed significant reduction of the retina thickness with aging, particularly that of the inner plexiform layer. To reveal changes between the age groups, we used immunohistochemistry against vesicular glutamate transporter 1 protein for photoreceptor and bipolar cell terminals, Brn3a for ganglion cells, calbindin 28 kDa for horizontal cells, parvalbumin for AII amacrines, protein kinase Cα for rod bipolar cells, tyrosine hydroxylase for dopaminergic cells, glial fibrillary acidic protein for glial cells, and peanut-agglutinin labeling for cones. The most significant decrease was observed in the density of photoreceptor and the ganglion cells in the aging process. By using immunocytochemistry and western blot technique, we observed that calbindin and vesicular glutamate transporter 1 protein staining do not change much with aging; tyrosine hydroxylase, parvalbumin and calretinin showed the highest immunoreactivity during the midlife period. Most interestingly, the level of glial fibrillary acidic protein also changes similarly to the previously named markers. Our results provide further evidence that protein content is modified at least in some cell populations of the rat retina, and the number of retinal cells declined with aging. We conclude that senescence alone may cause structural and functional damage in the retinal tissue.

Introduction
Aging is a natural component of life [Zahn and Kim, 2007; Tacutu et al., 2011]. Imbalance of the homeostasis originates from alteration of the environment causes permanent changes in the body with time. The central nervous system (CNS) is especially prone to losses of cells and proteins [Coleman and Flood, 1987; Majdi et al., 2009]. Since the CNS has a limited regenerative capacity, loss of function is inevitable over time [Lu et al., 2014; Tedeschi et al., 2017].
One of the best understood part of the vertebrate CNS is the retina, which is also sensitive for age-related changes [Samuel et al., 2011; Szabadi et al., 2015]. Since the retina is a laminarily organized tissue it is especially suitable for monitoring changes in cell numbers [Gao and Hollyfield, 1992; Curcio and Drucker, 1993; Panda-Jonas et al., 1995; Aggarwal et al., 2007; Parikh et al., 2007]. At the same time there are remarkable changes in the thickness of the distinct retinal layers during the aging process, especially in the outer nuclear layer (ONL) [Kuhrt et al., 2012]. It has been also described that the amplitudes of electroretinographic a- and b-waves decline with normal aging [Birch and Anderson, 1992; Trick et al., 1992; Freund et al., 2011]. The age-related increase in retinal area and the stability in cell number suggests that the surface density of cells decrease with age [Samuel et al., 2011]. Although approximately in the first 3 months (97 days in albino and 84 days in pigmented rats), these aspects change dramatically; the following phase is characterized by moderate alterations only [Nadal-Nicolas et al., 2018a]. Age-related ocular growth does not perfectly follow retinal area extension. Consequently, the retinal coverage of the ocular surface decreases during postnatal development. In rats, this coverage factor decreases from 73 to 41% [Kuhrt et al., 2012].

Age-related cell loss, among other causes, has been assumed to lead to mitochondrial damage [El-Sayyad et al., 2014; Gkotsi et al., 2014; Kam and Jeffery, 2015; Sivapatham and Tharam, 2017]. Deficits in the endogenous dopamine activity [Djamgoz et al., 1997] and/or a constant parainflammation [Xu et al., 2009; Arroba et al., 2018] have also been considered. The abovementioned authors have concluded that studying the effects of aging in experimental animals under a controlled environment and with similar genetic background can provide valuable information about the functional and anatomical impairment caused by senescence alone.

Selective effects of aging on particular components of neural circuitry, some of which may contribute to reduced visual function in old individuals, have also been studied. In rodents, aging causes morphological changes and/or loss of retinal ganglion cells (RGCs) and photoreceptors [Katz and Robison, 1986; Gao and Hollyfield, 1992; Semo et al., 2003; Cunea et al., 2014]. Other authors have not observed neuronal loss in the ganglion cell layer in aged animals while some studies have described that with age, the retina thins [Kim et al., 1996; Garcia-Ayuso et al., 2015; Nadal-Nicolas et al., 2018a].

In our previous reports, we have revealed the altered expression of cell type-specific marker proteins (tyrosine hydroxylase [TH], protein kinase Ca [PKCa], calbindin, calretinin, vesicular glutamate transporter 1 [vGLUT1], parvalbumin, glial fibrillary acidic protein [GFAP]) during the aging process of the retina in different aging models: degu [Szabadi et al., 2015], PACAP KO mouse [Kovacs-Valasek et al., 2017]. In this study, we use the same marker set for better understanding of these age-related changes in genetically homogenous population of rats older than 5 months and to determine the stage of age when these alterations become significant.

In order to reveal the retinal causes of age-related vision loss, we performed a 2-year-long study using genetically closely related female rats, which were kept under standard and identical conditions to limit genetic variability and exclude differences in environmental conditions.

Material and Methods

Animals
Wistar albino female rats originated from 2 sister litters were housed under identical conditions: in a temperature- (23°C) and light- (12/12h light/dark cycles) controlled room, with water and food ad libitum. Four separate cohorts were examined: 5-, 12-, 18-, and 24-month-old rats. Animals were sacrificed by an overdose of Forane anesthetic, and the eyes were immediately dissected in ice-cold phosphate buffered saline (PBS; pH 7.4).

Histology
Retinas were processed for histological analysis as previously described [Atlasz et al., 2010]. Briefly, the eye was enucleated, then immediately cut open. The eyecups of each animal were immersed in 1% glutaraldehyde-PFA overnight then dehydrated through ascending ethanol series. Finally, the tissue was embedded in Durcupan ACM resin (Merck, Budapest, Hungary). Resin sections were cut at 2 μm (Leica, Germany) within 1–2 mm distance from the optic nerve head and were stained with 1% toluidine blue (Sigma, Budapest, Hungary). The cross-section of the retina from outer limiting membrane to the inner limiting membrane and the width of individual retinal layers were measured using a Nikon Eclipse 80i microscope (×20 magnification objective) and the SPOT Basic program. At each age, we examined 15 microscopy frames (each frame measuring 500 μm) per eye, taken from a minimum of 4 animals. Values represent mean ± SEM.

Immunohistochemistry and Peanut-Agglutinin Labeling
The other eyecups of each animal were fixed in 4% PFA for 2 h at room temperature, cryoprotected in 15 and 30% sucrose solutions at 4°C. Retinal cryo-sections were washed with PBS 6 times for 5 min and blocked with normal goat serum for 1 h at room temperature. Then sections were incubated with either fluorescein isothiocyanate (FITC)-conjugated peanut-agglutinin (Vector Laboratories, Hungary; 1:1000) or primary antibodies (Table 1) overnight at room temperature and the next day with the corresponding secondary fluorescent antibodies for 2 h (Table 2). All sections
from different points of time were processed at once, using the same incubating solutions. Photographs were taken with an Olympus Fluorview FV-1000 Laser Confocal Scanning Microscope (Olympus, Japan), using the same settings (laser intensity of blue laser was 580 units and the green laser was 750 units; pinhole was 130 µm for green channel and 135 for red channel; optical section thickness was 1 µm) for one marker. The number of Brn3a-positive RGCs and cone terminals/100 μm retina length were also counted. Results are presented as mean ± SEM. Statistical comparisons were made using Student’s t test and one-way ANOVA (p < 0.05).

### Western Blot Analyses

Dissected retinas (n = 4 per age group) were homogenized in RIPA buffer (10 mM phosphate buffer, 15 mM NaCl, 0.1% SDS, 1% sodium-deoxycholate, 1% NP40, 2 mM EDTA, 2 μg/mL aprotinin, 0.5 μg/mL leupeptin, 2 mM sodium-vanadate, 20 mM sodium-fluoride, 0.5 mM DTT, and 1 mM PMSF). To remove insoluble material, homogenates were centrifuged at 13,000 rpm for 30 min at 4°C and the clear supernatants were stored at –80°C until use. Protein concentrations were determined with the BCA Protein Assay Kit (Thermo-Fisher, Budapest, Hungary) using BSA as a standard. Samples (20 μg protein/well) were electrophoresed on 4–12% NuPAGE SDS-polyacrylamide gels (Thermo-Fisher, Budapest, Hungary), then transferred onto PVDF membranes (Bio-Rad, Budapest, Hungary). Following transfer, the PVDF membranes were blocked with blocking solution containing 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, 5% non-fat dry milk, and 1% BSA for 60 min at room temperature. Membranes were sequentially probed overnight at 4°C with primary antibodies (Table 1), and the following day they were incubated with the appropriate secondary antibody (Table 2) for 2 h. Finally, blots were developed by incubating in enhanced chemiluminescence reagent (Advansta, San Jose, CA, USA) with Chemi Doc System (Bio-Rad, Budapest, Hungary). As a normalization control, GAPDH was used. Optical densities (OD) of the bands were normalized using the value found for the 12-month-old animals. Data are presented normalized OD ± SEM (n = 4; one-way ANOVA, Tukey-B Post hoc analysis; p < 0.05).

### Results

We carried out histological, immunohistochemical, and western blot analyses on rat retinas of different ages. Immunostaining for standard neurochemical markers (Brn3a for ganglion cells, peanut-agglutinin for cone photoreceptor cells, calbindin 28 kDa for horizontal cells, PKCα for rod bipolar cells, TH for dopaminergic and parvalbumin for AII amacrines, as well as calretinin for certain populations of amacrine and ganglion cells) revealed

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**Table 1.** Primary antibodies used in immunohistochemical and western blotting experiments

| Catalog No. | Primary antibodies | Raised in | Dilution immunohistochemical | Dilution western blotting | Company |
|-------------|-------------------|-----------|------------------------------|--------------------------|---------|
| SC8429     | anti-Brn3a Mouse  | 1:50      | –                            | –                        | Santa Cruz, USA |
| FL1071     | PNA (FITC conjugated) | 1:1,000   | –                            | –                        | VectorLab, UK |
| C9848      | anti-Calbindin Mouse | 1:1,000   | –                            | –                        | Sigma, Hungary |
| SC17804    | anti-PKCα Mouse   | 1:200     | –                            | –                        | Santa Cruz, USA |
| MAB318     | anti-TH Mouse     | 1:1,000   | –                            | –                        | Millipore, Hungary |
| P3088      | anti-Parvalbumin Mouse | 1:1,000  | –                            | –                        | Sigma, Hungary |
| PV235      | anti-Calretinin Mouse | 1:1,000   | –                            | –                        | Swant, USA |
| ab77822    | anti-vGLUT1 Rabbit | 1:500     | –                            | –                        | Abcam, UK |
| G9269      | anti-GFAP Rabbit  | 1:500     | –                            | –                        | Sigma, Hungary |
| G9545      | Anti-GAPDH Rabbit | –         | –                            | –                        | Sigma, Hungary |

**Table 2.** Secondary antibodies used in immunohistochemical and western blotting experiments

| Catalog number | Secondary antibodies for immunohistochemical | Raised in | Dilution | Company |
|----------------|-----------------------------------------------|-----------|----------|---------|
| A11001         | anti-mouse IgG conjugated with Alexa Fluor 488 | Goat      | 1:1,000  | Life Technologies, USA |
| A11011         | anti-rabbit IgG conjugated with Alexa Fluor 568 | Goat      | 1:1,000  | Life Technologies, USA |
| A11034         | anti-rabbit IgG conjugated with Alexa Fluor 488 | Goat      | 1:1,000  | Life Technologies, USA |

| Catalog number | Secondary antibodies for western blotting | Raised in | Dilution | Company |
|----------------|------------------------------------------|-----------|----------|---------|
| A3682          | anti-mouse IgG conjugated with HRP        | Goat      | 1:10,000 | Sigma, Hungary |
| 65-6120        | anti-rabbit IgG conjugated with HRP       | Goat      | 1:10,000 | Life Technologies, USA |
changes in neuronal structures between the age groups. Furthermore, we examined a synaptic protein, vGLUT1 and a metabolic stress-responsive glial marker protein, GFAP.

Morphometric analysis revealed significant decrease in the thickness of each retinal layer with age, especially in the inner plexiform layer (IPL) (shown in Fig. 1a, b). The most prominent reduction in cell number occurred in the ONL.
containing the photoreceptors which also appeared in lower densities at later ages (Fig. 2b). The density of ganglion cells also declined during the aging process (Fig. 2a).

Calcium-binding proteins are excellent markers of different subpopulation of neurons in the retina, and their expression show age-related changes [Papazafiri et al., 1995]. In our work, we examined 3 different calcium-binding proteins (calbindin, parvalbumin, calretinin) in the rat retina during the aging process. The sections at subsequent ages showed that the horizontal cell bodies and the outer plexiform layer (OPL) remained strongly stained by anti-calbindin antibodies. This staining decreased somewhat in intensity (Fig. 3a–d) although it showed less prominent difference during the aging process than the other calcium-binding proteins (see below). Western blots confirmed this pattern (Fig. 3e, f).

**Fig. 3.** Calbindin immunoreactivity in horizontal cells. Immunohistochemical labeling for 5 months (a), 12 months (b), 18 months (c), and 24 months (d), and western blot analysis (e, f). OPL, outer plexiform layer; INL, inner nuclear layer. Scale bar, 50 µm. Data are presented as mean ± SEM. ***p < 0.001 and **p < 0.01 compared to 5-month-old mature animals.
Rod bipolar cells can be selectively stained with antibody against PKCa and show decreased density in human and degu retina during aging [Aggarwal et al., 2007; Szabadfi et al., 2015]. We used PKCa antibodies for the detection of alterations of rod bipolar cells staining intensity at different age stages. Dendrites in the OPL, the cell bodies in the inner nuclear layer (INL) and axons terminating at the border of the IPL and ganglion cell layer were stained for PKCa (Fig. 4a–d). Labeling pattern does not show remarkable changes, but the intensity was constantly increasing (both the cell bodies and the terminals in the IPL) during aging, except the last time period when it remained constant. Western blotting confirmed these trends (Fig. 4e, f).

A decreased TH immunoreactivity has been revealed in aging human and PACAP KO mouse retina.
and Rees, 1997; Kovacs-Valasek et al., 2017]. Therefore, antibodies against TH were also used to study the level of this protein during the aging process in our present study. We revealed that TH expression reached a maximum at 12 months and has shown decreased levels afterward (Fig. 5a–d). Western blotting confirmed these results showing the highest protein level at 12 months (Fig. 5e, f).

**Fig. 5.** TH immunoreactivity in dopaminergic amacrine cells. Immunohistochemical labeling for 5 months (a), 12 months (b), 18 months (c), and 24 months (d), and western blot analysis (e, f). INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar, 50 μm. Data are presented as mean ± SEM. ***p < 0.001 compared to mature rats. ###p < 0.001 and ##p < 0.01 compared to 12-month-old animals.

Parvalbumin has long been considered as one of the most reliable markers of retinal cells [Endo et al., 1986; Sanna et al., 1990]. The cell bodies of AII amacrine cells in the IPL are intensively labeled by antibodies against parvalbumin in all examined age groups (Fig. 6a–d). Weakly immunoreactive structures, probably bipolar cells, were found in the INL, over and above immunore-
active amacrine cells. Those were detected in retinas of 12 and 18 months (Fig. 6b, c). Some ganglion cells showed moderate immunoreactivity at the same points of time (Fig. 6a–d). The intensity of the labeling was first increasing during aging (Fig. 6a–c) and declined after 18 months (compare Fig. 6c to Fig. 6d). This trend was confirmed by protein level measurements (Fig. 6e, f).

Calretinin has been localized to different neuron populations in the vertebrate retina [Pasteels et al., 1990]. Lately, it has been shown that the direction selective cholinergic cells contain calretinin in the rat retina [Gabriel and Witkovsky, 1998]. Besides the cholinergic cells, calretinin labeling was detected in some other amacrine cells and ganglion cell bodies and formed 3 distinct bands of the IPL (Fig. 7a–d). In the first year, immunoreactive
bands in the IPL were weaker (Fig. 7a, b) than at later ages (Fig. 7c, d). Subpopulations of amacrine and ganglion cells were also densely labeled. Amacrine cells and the IPL were very intense at 12 months (Fig. 7b). The highest immunoreactivity was seen already at 12 months of age both by immunostaining and western blot analysis (Fig. 7e, f). The labeling pattern did not change much during the examined time period.

Glutamate is a dominant excitatory neurotransmitter in the retina, and vesicular glutamate transporters take part in filling synaptic vesicles with this substance [Fyk-Kolodziej et al., 2004]. Among these transporters vGLUT1 is a major player, and its level changes significantly during the aging process [SzabadfI et al., 2015; Kovacs-Valasek et al., 2017]. In our experiments, the vGLUT1 protein expression did not show remarkable changes in the...
retinal tissue neither by immunocytochemistry (Fig. 8a–d) nor by western blotting (Fig. 8e, f). Among all, this marker proved to be the most constant during the aging process.

The GFAP is a stress indicator in the retina and during the senescence shows altered expression changes [Lundkvist et al., 2004]. In this study, GFAP staining has shown a remarkable change during the 24 months (Fig. 9a–d). While low at 5 months (Fig. 9a), strong elevation was seen at 12 and less intense increase at 18 and 24 months (Fig. 9a–d), and it declined again at 24 months (Fig. 9d). Interestingly, at older ages (18 and 24 months), both the immunocytochemical staining and the protein level of GFAP measured by western blotting occurred less intense and the blots showed fewer isoforms at 24 months than at earlier ages (Fig. 9e, f).

**Fig. 8.** vGLUT1 immunoreactivity in photoreceptor and bipolar cell terminals. Immunohistochemical labeling for 5 months (a), 12 months (b), 18 months (c), and 24 months (d), and western blot analysis (e, f). OPL, outer plexiform layer; IPL, inner plexiform layer. Scale bar, 50 µm.
Discussion

Aging phenotype varies between tissues and includes common hallmarks such as genomic and epigenetic instability, mitochondrial dysfunction, telomere attrition, and the accumulation of senescent cells [Deschenes and Chabot, 2017]. Any combination of the above processes will have basic influence on the speed of tissue deterioration and cell death. Since the retina is the metabolically most active organ in the mammalian body [Graymore, 1970; Kumagai, 1999; Nivison-Smith et al., 2015] and there is very limited regenerative capacity in the CNS, it is particularly prone to damage due to aging, which has been demonstrated by several authors [Gao and Holly-
In our study, retinal aging of genetically closely related female rat individuals (sister litters) kept under entirely identical conditions for a long period of time (up to 2 years) were studied utilizing standard cellular markers. Retinal Cell Type-Specific Observations

In this study, the investigated cell markers were listed in 3 different groups based on their expression pattern during the aging process. Some of these showed a steady decrease (peanut-agglutinin-labelled cones and Brn3a positive ganglion cells), others showed a midlife peak in their expression (TH, parvalbumin, calretinin, GFAP), while other proteins (calbindin, vGLUT1) do not change much during normal aging. Previous studies described the decrease of neuronal cell number in the aged retina [Weisse, 1995; Aggarwal et al., 2007; Samuel et al., 2011], and we also detected this trend for the majority of the markers (TH, vGlut1, Brn3a, calretinin, calbindin) latest after 12 months of age.

Nevertheless, neuron subsets are maintained in the aged retina, and cellular layers are organized properly, even if some reduction of the retina thickness and decreasing density of neurons in the cellular layers are evident in our study. Overall, these findings are in accordance with that reported for albino rats more than 3 months of age [Nadal-Nicolas et al., 2018a] and agree with the conclusions of our previous investigation where the retina in a 36-month-old Octodon degus was described as slightly loosened tissue structure both at light and electron microscopic levels.

In another series of experiments in mice, the responses of RGCs to visual features proved to be generally normal in most cases in aged individuals, only small differences in the physiology of old ganglion cells were seen [Samuel et al., 2011]. Other studies revealed the occurrence of tissue or even cell type-dependent differences in the autophagic pathways that are primarily affected by aging. In the case of the retina, these differences provide a plausible explanation for the specific pattern of sight loss with age [Rodriguez-Muela et al., 2013]. Together, these data indicate that aging may lead to decreased coverage of the visual field by ganglion cells leading to decreased visual acuity. This agrees with the finding that most ganglion cell dendrites also decreased [Samuel et al., 2011; Nadal-Nicolas et al., 2018a, b]. One would expect the dendritic field size to increase and cell densities to decline. If the cell densities remained the same during aging, one would expect a constant dendritic field size throughout life.

Gao and Hollyfield [1992] reported that cones are less vulnerable to loss than rods during aging. In this present study and in our previous work, we have shown that the cone density also decreased with aging [Kovacs-Valasek et al., 2017], and this age-related loss of photoreceptors was also reported in human [Gao and Hollyfield, 1992; Curcio and Drucker, 1993], rat [Cano et al., 1986; Nadal-Nicolas et al., 2018a, b], and mouse retina [Cunea et al., 2014]. The ONL thinning is less than expected based on cell counting data. This discrepancy can be explained by a gliotic scar forming in the ONL by Müller cell processes that partially compensates the volume loss.

Aging-associated disturbance of calcium homeostasis was described in many previous studies. They reported the age-related alterations of calcium-binding proteins and its different tendencies in CNS [Papazafiri et al., 1995; Kishimoto et al., 1998]. There is a general consensus about the decreasing tendency of calbindin and relatively stable parvalbumin and calretinin expression during normal aging. The previously described decreasing tendency of calbindin expression is supported in our results too, but in contrast to that, our study revealed a midlife peak during aging for parvalbumin and calretinin, but with a declining tendency afterward.

Age-related alterations were observed in the density of rod bipolar cells and the ribbon synapses of rods in O. degus [Szabadfí et al., 2015]. We also observed that dendrites of rod bipolar cells sprout well with aging. This alteration is also described in our previous work in aging PACAP KO mouse retina [Kovacs-Valasek et al., 2017]. This reorganization of dendrites is discussed in many other aging studies which describe the same phenomenon in mice and in human retinas [Liets et al., 2006; Elisieh et al., 2007; Terzibasi et al., 2009; Samuel et al., 2011]. The decreased number of rod bipolar cells in aging retina has also been reported in human tissue earlier [Ag-
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24-month-old rats [Jalenques et al., 1997]. In the human cochlear nuclei between age groups of 3-, 6-, 12-, and 24-month-old rats [Jalenques et al., 1997]. In the human retina, the GFAP level significantly increases during normal aging [Wu et al., 2003]. The age-related change of astrocyte density increased 3–9 months of age and decreased between 9 and 12 months in Wistar rats [Mansour et al., 2008]. At the same time, GFAP increase is often a sign of metabolic insults. This variation of GFAP expression during aging and metabolic retinal degenerations maybe due to changes in splicing. It is estimated that more than 50% of all age-associated alterations in alternative splicing are due to changes in the expression of splicing factors [Mazin et al., 2013]. This gives a good theoretical background for differential aging of individual cell types. Maybe glial cell aging is an important factor in the overall retinal tissue aging since glial cells are known to contribute to siphoning K+ from the extracellular space and to neutralization of reactive oxygen species that are generated as a consequence of inadequate microcirculation [Reichenbach and Bringmann, 2013].

Possible Mechanisms of Aging in Retinal Tissue

In most of the studies dealing with aging, both intrinsic and extrinsic factors are determined to contribute to the altered functional capabilities of cellular and molecular mechanism during the aging process. Three different types of physiological changes occur with aging: changes in cellular homeostasis, decrease in organ mass, and decline of functional reserve [Dodds et al., 2006]. Following this scheme, we found in our investigation that these age-related changes are especially well traceable and can be well documented in the aged retina (Fig. 10). Some aspects are also elucidated in our present study. For example, age-related metabolic changes contribute to the dysregulation of molecular processes and lead to a functional decline through altered cellular communication (blue rectangle in Fig. 10.) and a continuous slow parainflammation (red rectangle in Fig. 10.). Among the gradually decreasing biomarkers, researchers demonstrated a marked reduction of sirtuin 1 expression in aged retinal neurons [Luo et al., 2017].

Particularly interesting is GFAP from this aspect. Not only the quantity declines after a midlife peak, but also the number of isoforms seem to be less in old animals than in mature adults. A decrease in astrocyte availability has been described where some of the glial cells lose their GFAP content [Mansour et al., 2008]. The lower astrocyte density has been observed in adult rats compared to P18 age and significantly increased from P120 to P480 [Fernandez-Sanchez et al., 2015]. Interestingly, the same phenomenon has not been observed in 36-month-old O. degus. However, we have to note at this point that degu live much longer than rats and this aforementioned age maybe identical with the 18-month-old rats; therefore, the decline of GFAP content could not be seen yet. It is an especially important finding that this midlife peak tendency of GFAP expression was also described in the ventral cochlear nuclei between age groups of 3-, 6-, 12-, and 24-month-old rats [Jalenques et al., 1997]. In the human dystrophic retina, the GFAP level significantly increases during normal aging [Wu et al., 2003]. The age-related change of astrocyte density increased 3–9 months of age and decreased between 9 and 12 months in Wistar rats [Mansour et al., 2008]. At the same time, GFAP increase is often a sign of metabolic insults. This variation of GFAP expression during aging and metabolic retinal degenerations maybe due to changes in splicing. It is estimated that more than 50% of all age-associated alterations in alternative splicing are due to changes in the expression of splicing factors [Mazin et al., 2013]. This gives a good theoretical background for differential aging of individual cell types. Maybe glial cell aging is an important factor in the overall retinal tissue aging since glial cells are known to contribute to siphoning K+ from the extracellular space and to neutralization of reactive oxygen species that are generated as a consequence of inadequate microcirculation [Reichenbach and Bringmann, 2013].

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pathogenesis [Van Kirk et al., 2011], and there are endoge-
ous factors which can counteract both aging and reti-
nadamaging pathologies: NAP [Jehle et al., 2008]; PA-
CAP [Szabadfi et al., 2012]; NGF [Chen et al., 2015]; or
GLP-1 [Sampedro et al., 2019]. Monitoring of their ap-
propriate presence and maintenance of their physiologi-
ical expression during aging maybe the clue for slow aging
and longevity of proper retinal function.

The loss of proteostasis is a dominant factor in the ag-
ing process; almost all the proteins we examined in our
study decrease with old age (brown rectangle in Fig. 10)
[Rockstein and Brandt, 1963]. Although not directly ex-
amined in our study, there is evidence from the literature
that the decline in mitochondrial function and an in-
crease in ROS production damages the cell components
(orange rectangle in Fig. 10) and leads to senescence (gray
rectangle in Fig. 10) [Sena and Chandel, 2012]. It is also
well known that the retinal microvasculature also under-
goes remarkable age-related changes. Chan-Ling et al.
[2007] described the effect that caused the impairment of
the blood-retina barrier in aged rats compared to young
adults, and they found significantly increased vascular
permeability, decreased tight junction protein expres-
sion, and activation of major histocompatibility complex
class II-positive and phagocytic microglia [Chan-Ling et
al., 2007]. Deteriorated microcirculation maybe a first
step to a decline in mitochondrial quality and activity
which has been associated with normal aging [Sun et al.,
2016]. Besides, the specific inflammatory, neuronal and
microvascular genes altered with aging provide potential
molecular mechanisms for the well-documented func-
tional impairments and parainflammatory state of the
retina with advanced age. Additionally, these results sup-
port the hypothesis that diabetes and aging share some
common molecular alterations, but it is important to
know the patterns of gene expression are not identical
[Van Kirk et al., 2011; Gabriel, 2013]. These intrinsic and
extrinsic hallmarks of aging could be responsible for cel-
lar senescence, cell loss and decline of visual processing
capabilities.

Acknowledgement

The authors thank Alina Bolboaca for the excellent technical
assistance.
Statement of Ethics

All procedures abided the ethical permission approved by the University of Pécs (BA/02/2000-15,024/2011) and followed the ARVO guidelines regarding the protection of animals used for experimental and other scientific purposes.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Funding Sources

This work was supported by the Hungarian Brain Research Program KTIA_13_NAP-A-1/12-001, NKFIH grant (119289) and EFOP-3.6.2-16-2017-00,008.

Author Contributions

A.K.-V. wrote the manuscript, performed immunocytochemistry and western blots. E.P. wrote the manuscript, performed western blots, and collected literature. V.D. revised the manuscript, controlled sacrificing and dissection of animals, and supervised blotting. A.M. wrote the manuscript and performed western blots. G.S.Jr carried out confocal microscopy and figure planning. R.G. designed the study, supervised immunocytochemistry, wrote and revised the manuscript.

DOI: 10.1159/000515447
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