UNIVERSITY OF PÉCS

Doctoral School of Biology and Sportbiology

Investigation of normal aging in rat and PACAP-treated transgenic mouse retina

PhD Thesis

Etelka Pöstyéni

Supervisior:

Róbert Gábriel PhD, DSc

professor

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1. INTRODUCTION

1.1. The structure of the mammalian retina

The retina is a laminar, light-sensitive tissue located in the innermost layer of the eye. It is also part of the central nervous system**.** Among the main cell types of the retina, the photoreceptors, bipolar cells and ganglion cells are involved in the vertical information transmission pathway, with the axons of the ganglion cells forming the optic nerve that forwards information towards the higher visual centers. Interneurons, namely amacrine cells and horizontal cells, are involved in the formation of horizontal regulatory pathways. The cell bodies of these neurons and their synapses are organized into three nuclear and two plexiform layers in the retina, with the latter containing the actual synaptic connections between neurons. The layers from outside towards the inside are situated as follows: PE - pigment epithelium, ONL - outer nuclear layer, OPL - outer plexiform layer, INL - inner nuclear layer, IPL - inner plexiform layer; GCL- layer of ganglion cells. In the ONL, the cell bodies of the photoreceptors (cones, rods) can be found. In the INL, bipolar cells, horizontal cells, amacrine cells and Müller cells are present, while the ganglion cells and the "displaced" amacrine cells are located in the GCL. The information transfer between photoreceptors and bipolar cells takes place in the OPL, but horizontal cells can further modulate this stimulus transfer as inhibitory neurons. In the IPL, bipolar cells form synapses with ganglion cells and amacrine cells. The horizontal cells transmit information in the OPL to the bipolar cells using chemical synapses containing γ-aminobutyric acid (GABA). The amacrine cells, which form an extremely diverse group of retinal cells, receive information primarily from the bipolar cells and other amacrine cells, while they also have output towards the ganglion cells. Amacrine cells are extremely diverse both functionally and morphologically. Based on the arborization of their projections, there are amacrine cells with narrow, medium and wide dendritic fields. They have influence over the flow of information through inhibitory neurotransmitters such as GABA and glycine, moreover, GABAergic amacrine cells can also synthesize acetylcholine and dopamine. In addition to the listed cell groups, glial cells also play a significant role in maintaining the function and structure of the retina. There are three main types of glial cells in the retina: astrocytes and Müller cells (which belong to the group of macroglia), and microglia.

1.2. Aging and the aging retina

Aging is a complex, multifactorial process that encompasses a wide range of contributing factors and affects many different areas of life. Biological and chemical processes of aging can appear at molecular, cellular, and tissue levels and environmental, genetic, and epigenetic factors can also influence these processes (Khan et al., 2017). Aging has several main components and they exert their effects on the whole body. These aforementioned 7-10 overlapping processes were grouped using several experimental models and strategies. These components include metabolic and mitochondrial dysfunction, changes in intracellular communication, cell aging, telomere biology, genome instability, loss of proteostasis, stem cell depletion, epigenetic effects and changes in nutrient sensing. The causes of the aging process are classified according to their origin, such as primary (direct causes of aging-related damages), antagonistic (response reactions triggered by the damage); integrative (consequences of the previously mentioned response reaction) (López-Ótin et al., 2013). An extremely wide range of experimental models have been utilized to map the aging process, with mice and rats being the most commonly used models. The recent use of transgenic mouse contructs has, in addition, opened the way for experimental setups where the various expression patterns of different aging-related factors can be directly and systematically investigated. To understand the effects of natural aging on the retina is important because the retina itself is extremely vulnerable to the harmful changes caused by the aging process. This is, in part, due to the necessity to maintain its highly organized structure and the complexity of its synaptic network. In addition, the high metabolic activity and limited regenerative capacity also makes this tissue a sensitive target to damage. As a result, one of the most characteristic morphological change observable in the aging retina is a significant decrease in the thickness of the various retinal layers. The change in cell density and the alteration of synaptic connections can both be responsible for this structural alteration (Weisse et al., 1995; Samuel et al., 2011; Nadal et al., 2018; Mohamed et al., 2019). The morphological changes are, in turn, closely related to the functional differences, which can also be observed as age-related changes in the electroretinogram (Birch et al., 1992; DiLoreto et al., 1995; Freund et al., 2011; Samuel et al., 2011). Additional signs of aging were described in the retinal pigment epithelium and the blood vessels that supply the retina during the aging process.

1.3. Neuropeptides in the retina

Several neuropeptides are expressed in the mammalian retina and influence its physiological processes (Casini et al., 2005; Cervia and Casini, 2013; Cervia et al., 2019). One of these peptides is the pituitary adenylate cyclase-activating polypeptide (PACAP) that belongs to the VIP/secretin/glucagon peptide superfamily. We know of two naturally occuring forms, PACAP-38 and PACAP-27, with the former being the one typically expressed in vertebrates (Miyata et al., 1989; Arimura and Shioda, 1995; Vaudry et al., 2009). In the retina, PACAP is expressed by some cell bodies of the GCL and in amacrine and horizontal cells (Izumi et al., 2000; Dénes et al., 2014). In addition to its well-known complex effects described in individual disease models, its influence on the functional and structural changes in the aging retina has also been confirmed. In PACAP KO mice, the structural changes induced by aging are enhanced compared to other age-matched controls. In the case of somatostatin (SST), another prevalent neuropeptide, we can also distinguish between two biologically active forms (SST-14, SST-28). SST also exerts its pleiotropic effect through stationary G-protein coupled receptors (SST-1, SST-2, SST-3, SST-4, SST-5) that consists of seven transmembrane domains (Günther et al., 2018). Its presence in the retina has been confirmed in amacrine cells in the INL, and in displaced amacrine cells located in the GCL (Johnson et al., 2000; Cristiani et al., 2002). The decrease of its expression plays role, for example, in neurodegenerative processes observed in the diabetic retina (Carrasco et al., 2007; Hernández et al., 2014).

2. AIMS

The numerous physiological processes affected by aging, as well as the special vulnerability of the retina to them, raise several important and unanswered questions regarding the aging of the retina. From the scientific literature, it is well-known that the availability of certain neuropeptides in the body decreases with aging, leading to damages that occur at different systematic levels. The aims of my thesis are to examine the complex structural consequences of retinal aging by itself and in the context of the long-term effects facilitated by certain neuropeptides, in two different animal models. Accordingly:

1. We examined the structural changes of the retina during normal aging.

We have used various cell-specific and synaptic protein markers in immunohistochemistry and western blot procedures coupled with morphometric and morphological analysis, where we identified changes which appeared at the cellular level of the rat retina over time.

2. We investigated the effect of chronic PACAP treatments on the density of SST-containing and dopaminergic amacrine cells during the aging process.

Using immunohistochemical analysis, we investigated the changes of density in tyrosine hydroxylase-positive (TH, a marker of dopaminergic cells) and SST-positive cells as result of chronic PACAP treatment in a transgenic mouse model during aging. Additionally, we also aimed to identify any possible synergistic interactions between the peptides.

3. MATERIALS AND METHODS

3.1 Experimental animals and treatments

In our research, we have examined two models of aging. For the first one, we used Wistar albino rats and for the second one, C57Bl/6JTdTomato transgenic mice were used. The transgenic mice were produced at the Institue of Experimental Medicine, by crossing the sst/iresFlpo(Tm3) homozygous male with the GT(ROSA)26Sor_CAG/FSF_TdTomato (Tm) homozygous female. In the offspring, red fluorescent signal appears in the SST-producing cells. Animal handling and housing were reviewed and approved by the ethical committee of the University of Pécs (experiment permit numbers: BA02/2000-15024/2011, PE/EA/488-6/2021). The transgenic mice originated from the Institute of Experimental Medicine and were kept and cared for there. Intravitreal injections with PACAP were also carried out there. We separated the animals into three age groups (6 months, 12 months, 18 months). Individuals in the control group did not receive any treatment during the experiments. The eyes of the treated animals were injected intravitreally every three months under isoflurane-induced anaesthesia. The first injection was given at the age of 3 months. During the treatment, we injected PACAP1-38 (100 pmol) into one eye using a

Hamilton syringe (10 µl) with a 33G needle, and the same amount of physiological saline (0.9% NaCl) was injected into the other eye.

3.2. Morphological and morphometric analysis

After dissection of the eyes in the Wistar rat model, we prepared an eyecup preparation. The eyecup preparation was fixed in 1% glutaraldehyde-PFA until the next day. On the first day, it was washed in phosphate buffer and then dehydrated in an ascending series of alcohols. Afterwards, it was incubated in propylene oxide and then in a 1:1 mixture of resin and propylene oxide for 30 minutes, and then left in four-component Durcupan resin overnight at 4°C. On the third day, the preparations were embedded in resin blocks, which were allowed to polymerize for at least 36 hours at 56°C. Sections of the finished blocks were made with an ultramicrotome, then placed on a glass slide and stained with 1% toluidine blue before being covered with DPX coverslips. Photographs of retinal sections were taken using a CCD camera connected to a Nikon Eclipse 80i microscope, and morphological and morphometric analysis was also performed. Several parameters were examined using the SPOT Basic program (Spot Basic 4.04).

3.3. Immunohistochemical analysis

3.3.1. Normal aging model

The sections were prepared in the same way in both animal models. After fixation with 4% PFA, the retina was washed in phosphate buffer (containing NaCl) , then incubated in 15% and then 30% sucrose solutions until it sank. Retinal preparations were sectioned frozen using a cryostat device. The 10-12 µm sections were placed on slides. At the start of the immunohistochemical procedure, the sections were rehydrated and then blocked with Normal Goat Serum for 1 hour. Several different primary antibodies (anti-Brn3a, anti-PNA, anti-PKCα, anti-calbinid, anti-calretinin, anti-parvalbumin, anti-GFAP, anti-vGlut1, anti-TH) were used for the rat retinas. As a specific secondary antibody, Alexa Fluor "488" and "568" fluorescent markers were used in our normal aging rat model.

3.3.2. Transgenic mouse model

In order to validate our transgenic mouse model, SST and TH primary antibodies were used on retinal sections. Alexa Fluor "488" fluorescent secondary antibody was used, which was specific for the primary antibody. In the case of whole mount preparations of the transgenic mouse retina, after fixation with 4% PFA, the sclera was removed from the retinas in PBS buffered medium under a stereomicroscope. They were then washed in PBS buffer and blocked in TritonX-ABS. Wholemounts were incubated at 4°C with anti-TH antibody for 72 hours in a shaking machine. Afterwards, the preparations were washed in TritonX-PBS. They were then incubated with Alexa Fluor "488" secondary antibody for 24 hours at 4°C in a shaker. The photos were taken with an Olympus IX81 inverse platform type microscope, using an Olympus Fluorview FV-1000 confocal system, both in the normal and transgenic aging models.

3.4. Western blots

Tissues were homogenized in RIPA buffer. Then, the protein concentration was determined using a spectrophotometer with the BCA Protein Assay Kit and BSA as a standard. Protein electrophoresis was performed on a NuPage SDS polyacrylamide gel and then the proteins were transferred to a PVDF membrane. After the transfer, the membrane was dried at room temperature and then the membrane was incubated with the blocking solution at room temperature on a shaker. The membrane was then incubated with the primary antibody (anti-calbindin; anti-calretinin; anti-parvalbumin; anti-PKCα, anti-TH, anti-GFAP, anti-VGLUT1; anti-GAPDH). GAPDH was used as a normalization control. The next day, they were washed with TBST buffer, followed by incubation with horseradish peroxidase-conjugated secondary antibody. The optical density of the bands appearing in the case of different proteins was normalized to the values observed in the 12-month age group, and the relative protein expression was determined using GAPDH as an internal control. WesternBright ECL HRP substrate kit was used for detection and images were taken using the Chemi DOC System.

4. RESULTS

4.1. Normal aging model

4.1.1 Morphological and morphometric analysis

Thinning of the retinal layers was observed over time, especially with respect to the IPL. Among the nucleated cell layers, the ONL showed significant decrease. A gradual decrease of ganglion cell numbers in the GCL was also observed during aging. In addition, we observed a decrease in the number of cones in the ONL.

4.1.2 Immunohistochemical analysis

The activity of PKCα labelling (which marks bipolar cells) was initially weaker in the retinas of the younger age group, then remained almost unchanged in the last two age groups. The intensity of calbindin labelling, (which marks horizontal cells) decreased over time. The expressions of calretinin in the IPL was weaker in 5-month-old retinas than in older groups. The strength of parvalbumin staining initially increased and then decreased in the oldest age group. The TH level reached its maximum in the one-year-old age group, after that, it decreased. In our studies, vGlut1 labelling showed a slight decreasing trend during aging. The GFAP expression was low at the first age group, then appeared with a higher intensity in the one-year group and decreased again in the oldest group.

4.2. Effect of chronic PACAP treatment on the aging of the transgenic retina

4.2.1 Validation of the transgenic mouse model

To confirm the SST positivity of the fluorescent cells, double labelling was performed using an anti-SST antibody visualised with green fluorescence. In addition, we had to prove that the different amacrine cell populations could be clearly separated from each other, providing evidence that the TH immunoreactive and the SST-containing cells did not overlap. As a result of our double-staining experiment with anti-TH antibody, it was confirmed that the TH-positive amacrine cell population and the SST-positive cell population are separate from each other.

4.2.2 Changes of cell density after PACAP treatments in the retina

In the saline treated retinas, a slight decrease occurred in density of SST- and TH-positive cells with aging, which was observed both in the central and peripheral areas. The effect of PACAP treatment was first manifested in the 12-month age group, where there was an increase in the number of both TH- and SST-positive cells. During the regional comparison, we noticed that the peripheral areas were more affected by the increase in the number of both TH-positive and SST-positive cells than the central retinal areas.

5. SUMMARY

The natural aging process resulted in observable changes of retinal layer thickness and affected the density and appearance of the cells within.

1. In our morphological and morphometric results, we proved the decrease of retinal layer thickness on various occasions, which affected the the IPL and the ONL most notably. Furthermore, we were able to highlight a significant decrease of the number of ganglion cells and cones during aging.

2. In our immunohistochemical studies, we have shown that with the markers we used three groups of cells can be defined based on their expression patterns: those showing decreasing expression over time (PNA, Brn3a), those showing a midlife peak (TH, parvalbumin, calretinin, GFAP) and those showing only a slight change as the aging progresses (calbindin, PKCα, vGlut1).

3. The density of SST-positive amacrine and TH-positive dopaminergic cells decreased in the retinas of transgenic mice over time, which we could reduce by intravitreal injection of PACAP. Both SST and PACAP belong to a broader family of neuropeptides and exert their effects through G-protein-coupled receptors, and for that their intracellular pathways overlap at many points, making an enhanced protection possible.

4. Based on well-known factors from literature and our experimental results, we have drawn up the schematic mechanisms of retinal aging.

5. In our experiments, we investigated the relationship between two neuropeptides using a transgenic model. Based on our results, we propose that this approach can be useful in the future for designing combined treatment with simultaneous application of different neuropeptides.

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7. Publications

7.1. Publications related to the thesis

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