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Exosomes and alpha-synuclein within retina from autophagy to protein spreading in neurodegeneration

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A B S T R A C T

In the course of age-related macular degeneration (AMD) as well as in multiple retinal disorders protein aggregates are described at various level in the retina. In AMD this fills the space between retinal pigment epithelium (RPE) in the form of drusen, which contain amyloid and other protein aggregates along with lipids. Nonetheless, in very advanced stages of AMD, as well as in other retinal pathologies and early on in retinitis pigmentosa, a number of neuronal inclusions, which stain for α-synuclein spreads all over the retinal layers. Thus, an early or later defect in the clearance of α-synuclein may represent a final common pathway to these phenomena. The physiological clearance of α-synuclein is provided by the autophagy machinery starting at the level of the RPE and occurring throughout the retina. Such a process is also involved in the clearance of melanin-dependent toxic metabolites under the effects of different wavelength and the stimulatory activity of the sympathetic nervous system. In search for the occurrence of these culprits, here we report the presence of α-synuclein in the retina combined with exosomal detection to document the presence of a α-synuclein spreading apparatus. This was correlated with the occurrence of autophagy markers throughout retinal layers, along with sympathetic innervation, which in turn was related to melanin content.

Key words

Age-related macular degeneration • Neurodegeneration • Autophagy • Protein aggregation • Exosomes • Pigment epithelium

Introduction

Occurrence of protein aggregates in the retina is a common finding in pathological conditions featured by degeneration of various retinal layers and altered retinal structure. This is the hallmark of age-related macular degeneration (AMD, Jager et al., 2008), where drusen formed by debris of proteinaceous material and lipids occur as aggregates in the outer retina between the retinal pigment epithelium (RPE) and the choroid (see representative Figure 1, Bergen et al., 2019; Pfeiffer et al., 2020; Pinelli et al., 2020a, 2020b). These may also extend as pseudo-drusen between the RPE and the outer segment of photoreceptors. Inclusions are not unique for AMS as they characterize retinitis pigmentosa, where protein aggregates, which specifically contain α-synuclein are described (Pfeiffer et al., 2020). A similar pattern occurs during post-traumatic retinal degeneration (Pfeiffer et al., 2020). Despite early anatomical features, which remain distinct for various kinds of retinal degeneration, at later stages the process evolves following a convergent final common pathway where distinct features typical and hallmarking each type of retinal degeneration cannot be distinguished any longer, and a frank synucleinopathy spreads along various retinal layers (Pfeiffer et al., 2020). Thus, we may assume that

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early pathology remains distinct and it is unique for various kinds of retinal degeneration, however, during advanced disease stages the pathology evolves in a way, which resembles proteinopathies being described in various degenerative disorders affecting the CNS. In detail, the retinal pathology matures towards a frank synucleinopathy, which affects various retinal neurons (Pfeiffer et al., 2020). Such a phenomenon is reminiscent of late stages during the course of classic degenerative disorders such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (Pfeiffer et al., 2020). In fact, previous manuscripts document such a pathological contamination between various disorders, which occurs as the consequence of a metabolic defect in protein handling. This is typical of neurodegenerative disorders (Fornai et al., 2002; Fornai et al., 2003a; 2003b; Fornai et al., 2005; Pfeiffer et al., 2020), where α-synuclein deposition is evident during Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), multiple system atrophy (MSA), diffuse Lewy body dementia (DLBD), Huntington disease (HD), and specific types of spinocerebellar atrophy (SCAs). In recent manuscripts it was hypothesized that such a disease contamination may already be present early, during a pre-clinical stage, especially concerning specific disease phenotypes (Fornai et al., 2005; Ferrucci et al., 2013; Pasquali et al., 2014; Gambardella et al., 2017; Giorgi et al., 2017; Silani et al., 2017; Limanaqi et al., 2019; Limanaqi et al., 2020). This was early postulated in a pioneer manuscript suggesting a prion-like spreading of neurodegeneration within the CNS, which may occur as the consequence of a metabolic defect in the clearance of key proteins, all featuring a prion-like structure (Fornai et al., 2006). Thus, it was originally postulated that disease spreading within the CNS may be the consequence of a process, which extends beyond infective or genetic mechanisms to recruit circuit-specific metabolic alterations in protein handling (Fornai et al., 2006). This hypothesis was confirmed a couple of years later in PD patients, by showing the spreading of the prionoid protein α-synuclein from the striatum of a PD patient within neighbouring grafted dopamine (DA) producing cells (Kordower et al., 2008a, 2008b). These findings led Olanow and Prusiner to postulate more specifically the occurrence of a prion-like spreading of α-synuclein to extend neurodegeneration in PD (Olanow and Prusiner, 2009). Despite some controversies (Olanow and McNaught, 2011; Olanow and Isacson, 2012; Olanow and Brundin, 2013; Olanow, 2014; Shapira et al., 2014), which are addressed in the discussion of the present manuscript, these data are confirmed even by recent reports. In fact, a causal time-dependency between neuro-inflammation and α-synuclein diffusion was recently indicated within a grafted tissue of a PD patient (Olanow et al., 2019). The spreading of neurodegeneration was extended to other disorders such as ALS (Fornai et al., 2011; Silani et al., 2011), progressive autonomic failure (PAF, Natale et al., 2013), neuroenteric dysfunction (Natale et al., 2011), anterior horn degeneration in the spinal cord (Ferrucci et al., 2017) and even in the maturation of seizure-induced brain circuitries (Orzi et al., 2013). In keeping with retinal degeneration, the merging in pathology appeared only to concern the final stage of different subtypes of the same retinal disorder (such as the mixed nature of wet and dry age-related macular degeneration, AMD, Pinelli et al., 2020b). Later on, it was described between distinct retinal disorders. This merging neuropathology, both when occurring within subtypes of a single retinal degenerative disorder and when it extends to various retinal degenerations (i.e. AMD and retinitis pigmentosa) indicates that a process leading to maladaptive maturation is taking place as suggested by Pfeiffer et al. (2020). Concerning this point in a concomitant paper (Pinelli et al., 2020b) an abnormal autophagy-dependent protein spreading was hypothesized, which invades retinal layers based on exosomes or exosome-like cell-to-cell transmission. This is in line with the need of the cell to get reed of toxic/overwhelming cargoes when cell clearing systems are impaired (Zhan and Schekman, 2013). In keeping with this, in their elegant paper Authors defined such a phenomenon as "unconventional secretions, unconventional solutions" (Zhang and Schekman, 2013). When translated at retinal level this concept leads the main cause for visual impairment within early metabolic defects of the retinal pigment epithelium (RPE) as already postulated by Jones et al. (2016). In fact, metabolic alterations of RPE cells may predict progression of disease (Jones et al., 2016). While this concept was early expressed as a general

strategy in the context of cell-to-cell communication in a variety of physiological and pathological conditions, the occurrence of cell-to-cell spreading of protein cargoes at the level of the retina remains non-investigated. In this way, the eye, once again, shows its remarkable value as "a window in the brain", which allows to study in a sensory organ the intimate phenomena which otherwise are considered to be typical of exquisitely hidden areas of the brain. These recent insight into a merging retinal pathology mimicking neurodegenerative disorders prompted us to challenge the retinal layers in baseline conditions for the presence of those elements which characterize the seeds of pathophysiology in spreading degenerative disorders of the CNS. Therefore, in the present manuscript we looked on the presence of the secretory apparatus underlying extracellular release of α-synuclein in baseline conditions in the retina, by questioning the presence and placement of the exosome-related marker TSG101 along retinal layers in combination with the occurrence of the prion-like protein α-synuclein in the very same retinal layers. This analysis was carried out to get a formal evidence for a potential exosome-mediated transmission within various layers of the retina, even in baseline conditions. In fact, α-synuclein is the prototypical protein, which undergoes cell-to-cell spreading while being the hallmark of late stages of retinal degeneration. This staining was carried out in baseline conditions to probe the presence of those seeds (exosomes and α-synuclein immunostaining), which are supposed to exaggerate their operations during the spreading of degenerative phenomena along retinal layers, as they do in classic neurodegenerative diseases.

In the same baseline conditions, the occurrence throughout retinal layers of the classic autophagy marker MAP-LC3β was documented. In fact, the most common metabolic defect leading to the formation of neuronal inclusions and proteinaceous aggregates all over the brain consists in an acquired or inherited defect in the autophagy machinery (Madeo et al., 2009), which is grossly marked by the typical protein LC3. Again, due to a potential modulatory effect of catecholamine metabolism under the effects of abnormal light activation (Pinelli et al., 2000a, 200b) a concomitant staining for tyrosine hydroxylase (TH, the rate limiting step in catecholamine biosynthesis) immunoreactivity was carried out. In fact, the synthesis of melanin in the RPE and suprachoroidal layers is under the influence of TH positive sympathetic fibers (Koka and Patel, 2021). In turn the presence of melanin is key in producing quinone-derived reactive oxidative species which binds and oxidize cysteinyl containing amino acids leading to protein misfolding as in the case of α-synuclein (Conway et al., 2001). In fact, melanin is structurally associated with pathological hallmarks of retinal degeneration (Yamanari and Mase, 2020).

Methods

Animals

Six adult male Wistar rats, weighting 300 g and six C57BL6J male mice (23 g) (Harlan, S. Pietro al Natisone (UD) Italy), were used for the study. The animals were housed for one week in paired cage, at light/darkness cycles of 12 hours and with free access to food and beverage and adequate measures were taken to minimize animal pain and discomfort. The European rules (CEE 86/609) were followed for what concerns animal housing, health and experimentation. The retinal samples used for this study were dissected out in the course of old experiments carried out between 2011 and 2013 and they were harvested in our bank of mouse and rat tissues (in the present case C57Bl6 mice, and Wistar rats) previously used in a project approved by the Italian Ministry of Health (authorization number 267/2011-B).

Histology and immunohistochemistry

The storage of the retina was carried out by dissecting the posterior segment of the eye, where the retina was attached, this segment was immediately placed in a Carnoy solution containing ethyl alcohol (60%), acetic acid (10%), and chloroform (30%), and 20 hours later, it was placed in 70% ethanol until paraffin inclusion to be stored for future analysis up to the present study when it was further analysed and processed. At the time of present morphological investigations the retina from both eyes of rats and mice were cut into 10 μm serial sagittal sections (through the medio-lateral extent of the posterior eyeball) and it was used for histological and immunohistochemical analysis. For histochemistry sections were de-waxed and processed for staining with haematoxylin & eosin (H&E).

Tab. I - Source, specificity and concentration of antibodies used in the study.

Antibody	Distributor	Catalog Number	RRID	Concentration
Monoclonal mouse anti-TH	Sigma Aldrich, Milan, Italy	Cod.T1299	AB_477560	1:100
Biotinylated horse anti-mouse IgG (H+L)	Vector lab. Burlingame, CA, U.S.A.	Cod. BA-2000	AB_2313581	1:200
anti α-synuclein	Sigma Aldrich, Milan, Italy	Cod. SAB4502828	AB_10746104;	1:100
Biotinylated Goat anti-rabbit (H+L)	Merck Millipore, Burlington, MA, U.S.A.	Cod. 401393	AB_437797	1:2000
Monoclonal rabbit anti-TSG101	Abcam, Cambrige, U.K.	Cod. ab125011	AB_10974262	1:100
Polyclonal rabbit anti-MAP LC3 Beta	Santa Cruz biotechnology, Dallas, TX, U.S.A.	Cod. sc-28266	AB_2137719	1:100
Alexa Fluor 488 Donkey anti-rabbit	Thermo Fisher Scientific, Waltham, MA, U.S.A.	Cod. A21206	AB_2535792	1:100

In order to investigate the α-synuclein, TSG101, Tyrosine hydroxylase, and MAP-LC3β amount and localization, retinal specimens were treated for immunohistochemistry and immunofluorescence. Tissue sections were de-waxed and antigen retrieval was performed with citrate buffer pH 6.0 for 10 min. Sections were incubated with 0.1% Triton X-100 (Sigma Aldrich, Milan, Italy) for 15 min, soaked in 3% hydrogen peroxide to block endogenous peroxidase activity (immunoperoxidase) and they were treated with normal serum for 1 h (10% in PBS). Then, sections were incubated overnight with primary antibody (see Table I) and then for 1 h with secondary biotin-coupled anti-rabbit/mouse IgG (1:200; Vector Laboratories, Burlingame, USA) or Alexa Fluor 488-coupled anti rabbit (section were counterstained with DAPI in immunofluorescence figures, see Table I). Negative control sections were obtained by repeating the same procedures but primary antibodies. Peroxidase activity was detected by using a solution containing 0.04% of 3,3'-diaminobenzidine-tetrahydrochloride (DAB; Sigma Aldrich) pH 7.6, for 3 min at room temperature (RT). The stained sections were dehydrated, cleared and cover slipped with Micro-mount (Diapath, Martinengo, BG, Italy).

Densitometric Analysis of MAP-LC3β immunoreactivity in the retina

Retinal MAP-LC3β immunoreactivity was semi-quantified by measuring relative optical densities. The retina was sectioned for the entire medio-lateral extent in 280 μm distant medio-lateral serial sections. Each section was analyzed by measuring the optical density for five microscopy fields and five retinal ganglion cells for each animal. Images were acquired at low magnification (× 2.5). Analysis was carried out by assessing the intensity of the cells, compared with a background value (the optical density measured in unlabelled areas present in the section, i.e. sclera) by using Zeiss Axio Imager M1 microscope equipped with a motorized stage and focus control system (Zeta axis), and with a digital video camera. Results are given as the mean±S.E.M. for each animal. Two sample T-test (H_0: $\mu_1=\mu_2$) was used for statistical comparison of collected data. Hypothesis H_0 was rejected when $P \leq 0.05$.

Results

Histochemistry

Figure 1 shows a representative picture from a rat retina where, following staining with H&E all layers were identified as reported in Figure 1. Such an introductory image was selected from a rat retina since the albino Wistar rats do not contain melanin both in the choroid *lamina fusca* (supra-choroidal layer) and retinal pigment epithelium (RPE). This allows better discerning the various cells in the external retinal segment and the choroid layer. Therefore, the names of identified layers of the retina were easily matching with evident cells aggregates. When the H&E staining from an albino rat is compared with the H&E staining in a pigmented C57 Black mouse the different amount of melanin in the choroid and the RPE is evident as reported in Figure 2. Such a remarkable difference is not a mere histological variant. In fact, occurrence of retinal inclusions is very different

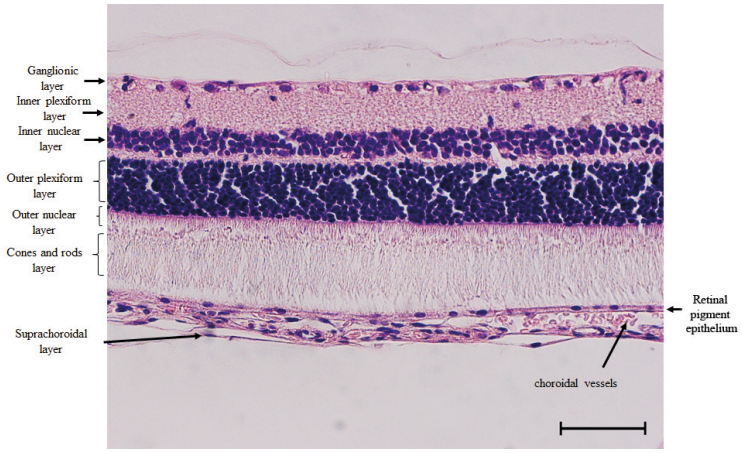

Fig. 1. - Hematoxylin & Eosin (H&E) staining of the retina from Wistar albino rats.
The retinal layers are easily recognized and cells of the outer retina can be easily identified due to the absence of melanin. All retinal layers are reported.
Scale bar=100 µm.

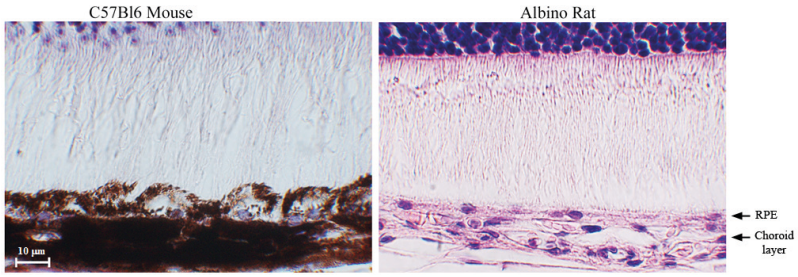

Fig. 2. - Comparison of the retina from albino and pigmented species.
The haematoxylin & eosin (H&E) staining allows a clear cut separation of the retinal layer from an albino Wistar rat, while the confounding presence of melanin which extends from the RPE throughout the external segment of photoreceptor obscures the detection of cells in the external retina. The presence of melanin in a pigmented C57 Black mouse stimulates a thicker RPE which sends off cellular processes towards the photoreceptors, which contrasts with the flat RPE cells occurring within albino rat.
Scale bar=10 µm.

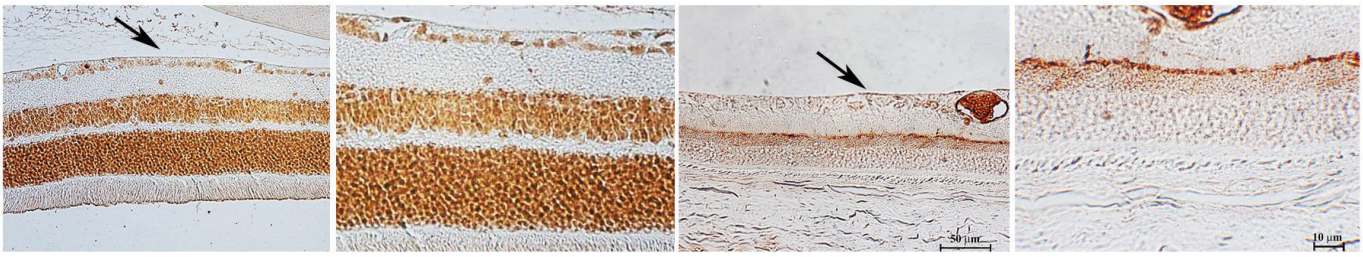

Fig. 3. - Comparison of retinal TH immunostaining from albino and pigmented species.
In line with data showing a powerful stimulation of the sympathetic nervous system in melanin production, the retina from a pigmented C57 Black mouse is densely innervated by TH positive fibers. These form two dense network in the outer and inner nuclear layer. In contrast, in the retina of an albino Wistar rat TH immunostaining only occurs within scattered perikaria mostly in the plexiform layer.
Low magnification Scale bar=50 µm; High magnification Scale bar=10 µm.

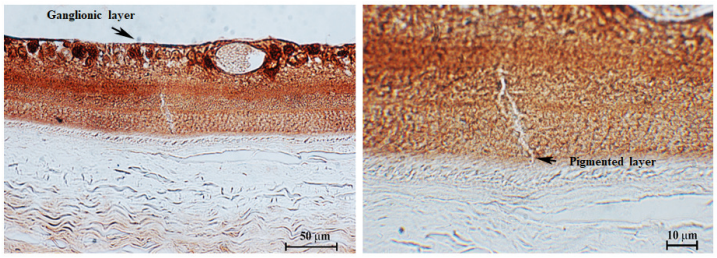

Fig. 4. - Representative immunostaining of MAP-LC3β.
The autophagy marker LC3 stains profusely the retina, which is in line with the seminal role of the autophagy machinery at retinal level and the detrimental degenerative effects, which are produced in the retina when autophagy is impaired.
Low magnification Scale bar=50 µm; High magnification Scale bar=10 µm.

in the albino retina including humans, which suggests a significant contribution of catecholamine derivatives forming melanin as well as the tyrosine hydroxylase (TH) activity in the retina itself, which is supposed to contribute to retinal degeneration (Pinelli et al., 2020b). Histologic evaluation of retina from pigmented mouse does not allow to detect the external segment of photoreceptors, unless using melanin bleaching and tissue clearing (Lee and Kim, 2021), which being entrapped between digitate protrusions of the RPE are not visible due to the diffusion of the melanin staining (Figure 2A). In contrast, the RPE is detected very well in the albino retina where a single cell layer of quite flattened cells surrounds externally the external segment of photoreceptors, which is well evident (Figure 2A). It is noticeable that where the pigment is not produced the RPE cells do not project cell protrusions to interpose between the external segment of photoreceptors. In fact, there is no need to prevent light to pass from one receptor to another since no pigment can counteract such a diffusion. In this way, it is likely that the synthesis of melanin within RPE also represents a stimulus for developing and extruding cell processes, which in fact do not occur in the albino retina (Figure 2A). Again, the shape of the RPE cells tend to be more flattened in the albino retina compared with the pigmented retina, which might simply represent the consequence of a lack of cytosolic content within the albino RPE, where melanin fills and enlarge the cytosol of RPE cells in the pigmented retina. This is not surprising when considering the cell process, which develops from the cell bodies of melanocytes in the skin and the occurrence of large melanosomes within the cytosol of these cells.

Representative TH immuno-peroxidase

Occurrence of TH immunostaining markedly differs between albino and pigmented rodents. In wist rats TH is much more restricted compared with previous antigens and it is marked only within the inner plexiform layers where, amongst amacrine cells some are known to produce catecholamines as a neurotransmitter. In this cells a specific TH immunostaining is detectable within albino rats which lack TH within all retinal layers but the inner plexiform to a small extent (Figure 3). This is in sharp contrast with pigmented C57Black mice where TH immunostaining is abundant in the retina as much as the melanin content. In fact, at least two layers possess a thick background of TH immunostaining which include both the outer and the inner nuclear layer. In this way, the pattern of TH immunostaining markedly differs between albino and pigmented rodents, where in C57 black mice the TH immunostaining is reciprocal compared with α-synuclein or exosome immunostaining, which never occurs neither in the outer nor in the inner nuclear level, contrasting with the plexiform layers. Within the inner plexiform layer, scattered TH positive cells are present in pigmented mice, although this is less evident compared with albino Wistar rats. The pattern of TH immunostaining goes along with the occurrence of melanin within the retina and it confirms a powerful effect of the sympathetic innervation in inducing melanin formation. Nonetheless, the inner and outer nuclear level, despite being fully crowded of TH positive fibers do not possess detectable melanin.

Placement and amount of retinal MAP-LC3β.

As predicted by a strong ongoing retinal autophagy activity, the marker of autophagy progression MAP-LC3β is detectable within all retinal layers, starting at the level of RPE with a slight decrease at the level of photoreceptors and an increase in the internal retina where the highest amount is detected within retinal ganglion cells (Figure 4). In detail, these cells are fully stained by LC3 immuno-peroxidase and the ganglion cells, which are mostly stained seem to correspond to the greatest types (M type ganglion cells). Within these various retinal layers only the retinal ganglion cells possess the whole cell body which intensely stains for LC3. Within other retinal layers the LC3 staining provides a strong background which does not allow to distinguish cell bodies clearly. Only small cell bodies can be distinguished within RPE and within the amacrin cell layer. As shown in Figure 6, when comparing the amount of LC3 staining within ganglion cells with the other retinal layer a marked difference exists showing that the ganglion cell layer possesses the highest LC3 intensity (graphs of Figure 5).

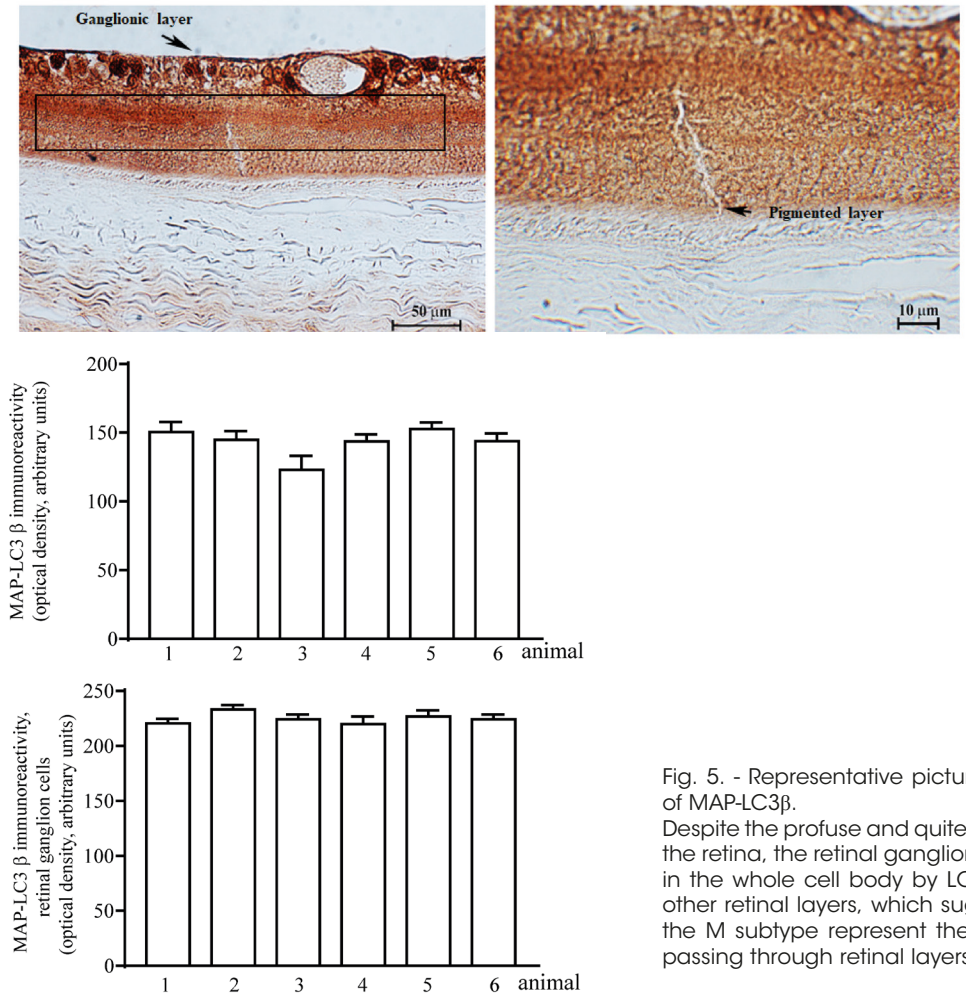

Fig. 5. - Representative pictures and densitometric counts of MAP-LC3β.
Despite the profuse and quite ubiquitous presence of LC3 in the retina, the retinal ganglion cells are remarkably stained in the whole cell body by LC3 antibodies compared with other retinal layers, which suggests how these cells mostly the M subtype represent the convergency of all cargoes passing through retinal layers by cell to cell transmission.

Immunostaining for α-synuclein and exosomes marker (TSG101)

The staining for the protein α-synuclein within the retina reveal a quite widespread signal in various retinal layers, which is better defined by the counterstaining with DAPI. As shown in Figure 6, the presence of α-synuclein was mostly evident as fluorescent puncta within the retinal pigment epithelium, being also abundantly detected within photoreceptors, although following a quite widespread pattern (Figure 6A). Both the outer and inner nuclear layers (evident by an intense blue fluorescence due to DAPI staining did not contain α-synuclein), which again was evident in the external and internal plexiform layers, which is consistent with the synaptic placement of this protein (Schlüter et al., 2003). A punctate pattern of α-synuclein fluorescence, which was mimicking what described within RPE was present in the retinal ganglion cells (Figure 6B), although in this layer the presence of α-synuclein was much more scattered. Within outer and inner plexiform layers α-synuclein immunofluorescence was detected according to a widespread pattern without specific puncta which remain mostly evident in the RPE compared with all retinal layers. The staining for the exosome specific marker TSG101 (Figure 6B) in the retina was very similar to that described for α-synuclein. In fact, the pattern of TSG101 immunofluorescence shows a widespread presence throughout the various layers of the mouse pigmented retina with an abundant amount in the RPE, the photoreceptors, the outer and inner plexiform layer along with detectable immunofluorescence in the ganglionic cell layer with no detectable fluorescence in the outer and inner nuclear layers (Figure 6B). Again, occurrence of TSG101 as much as synuclein is more evident where synaptic activity takes place, apart from the presence in the thin RPE.

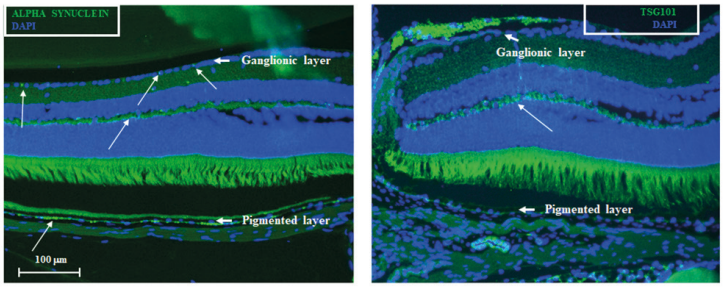

Fig. 6 - Representative immunostaining of α-synuclein and TSG101.
Overlapping pattern of α-synuclein immunostaining with the exosomal marker TSG101, which is evident in all retinal layers but the outer and inner nuclear layer. A finding which reciprocate the pattern of TH immunostaining. Scale bar = 100 µm.

Discussion

The role or RPE in AMD is key in fostering and/or counteracting the disease course and the process of inflammation. The structure of RPE markedly depends on the presence of melanin as shown in the present manuscript and described in other studies. In fact, here light microscopy evidence is provided showing a flat cell layer forming the RPE in the albino retina in the absence of cell protrusion intermingled between the outer segment of photoreceptors. The flatness and absence of cell processes in the albino RPE is likely to depend on the phenotype of melanin containing cells, which derives from neural crest and develop cell processes just like melanocyte of the skin. In the RPE these processes are key to seal the outer segment of each photoreceptor and for preventing the light to pass from one cell to the other through its lateral domain. Just like melanocyte RPE cells contain melanin bodies which fills the cytosol and they are transported along cell processes to spread the melanin content to the whole volume of the cell. Thus, the sympathetic-dependent development of a melanised RPE produces considerable effects concerning the cell size and shape and alter the biochemical properties of the RPE. In fact, the occurrence of melanin markedly alters the fine biochemistry of the cell and the buffering of reactive oxygen species and free radicals, including those produced by specific wavelengths. Again, melanin is key in binding with misfolded proteins and may be critical in the spreading of degeneration when the metabolic activity of the RPE is altered. It is remarkable that the synthesis of melanin within RPE is strongly dependent on the sympathetic activity. In fact, when comparing the amount of TH immuno-positive structures there is an impressive amount of TH immunostaining within pigmented mouse compared with the albino rat, which lend substance to a powerful role of sympathetic fibers in producing melanin. This also produces marked morphological effects within RPE. In fact, within melanised retina the structure of RPE cannot be detected, unless removing melanin as previously emphasized. The occurrence of melanin provides an increased thickness of RPE cells and, being also present in the external layer of the choroid, it impedes the visualization of the choroid retinal border. Such a difference is remarkable and it goes beyond a mere histological finding since it extends to the significance of melanin in modulating the occurrence of intra- and extra-cellular inclusions. In fact, the presence of melanin is key to bind misfolded proteins and oxidized lipids, which alter the process of drusen deposition in AMD. In fact, these Authors could not describe drusen from the retina of albino patients, which is in line with the report of a flat retina in the presence of degeneration reported in albino patients.

The occurrence of melanin within RPE and choroid lamina fusca strongly depends on the sympathetic innervation, which extends the modulation of melanin-dependent retinal degeneration to the amount of sympathetic innervation of the retina. The double-edged role of melanin in retinal degeneration is fascinating, in fact from one hand it is related to the occurrence of retinal inclusions characterizing various forms of retinal degeneration such as drusen in AMD. On the other hand some findings highlight a potential benefit of melanin in reducing the cell damage which occurs in the proximity of drusen in AMD (Herrera and Beeraka, 2020). This is not surprising when considering the classic question whether entrapping proteinaceous and lipidic material within synuclein inclusions is a buffer to counteract the spreading of degeneration or instead it is by itself a detrimental factor for neuronal survival.

Here again, the eye configures as the gateway to comprehend the significant of neuronal inclusions in neurodegenerative disorders. In fact, the presence of a melanized RPE may be considered as a defense to the photo-oxidation occurring in the retina (Mahendra and Tan, 2020). On the other hand, the wavelength of light sorts different effects on the melanin structure, which may lead to opposite outcome for the survival of retinal cells. It is a paradigm the occurrence of flat retinal damage in albino eyes with no drusen or essudates (Sharma et al., 2013), which suggests that drusen by itself as much as other inclusions do not produce the damage and their presence is rather the effect of an attempt to buffer oxidative and inflammatory species (Pinelli et al., 2020b). Thus, it remains a matter of debate the complex role of melanin in counteracting or fostering retinal degenerations as much as the role of light as a whole appears to be gross to be solved in a one way effect and it rather deserves an analytical approach to address the specific effects of discrete ranges of wave length.

In any case, due to its high melanin content the RPE plays a key role in retinal physiology and degeneration. Even the occurrence of inflammation in the retina is grounded on the activation of inflammasome and immunoproteasome within RPE. Such a key role was recently emphasized by a number of papers (Pinelli et al., 2020a, 2020b) leading to hypothesize the use of specific siRNA to occlude the expression of inflammatory protein produced by the RPE in the shift towards wet AMD (Ramsay et al., 2020). Although such an approach is limited by the hurdles opposing to siRNA penetration within RPE including the melanosomal sequestration of siRNA (Ramsay et al., 2020). The approach to substitute a damaged or dysfunctional RPE is not just dictated by the need to reinstate the seminal functions played by RPE in the retina but it also concerns the chance to erase the pathogenic role of a dysfunctional RPE. Thus, an alternative approach is proposed consisting in delivering IPSC derived RPE cells with the aim to substitute through endogenous stem cells differentiated into RPE the defective activity of RPE itself (Matsuzaki et al., 2020). Since endogenous stem cells occur at the outer border of "*ora serrata*", in the so marginal zone of the ciliary body also known as "*ora terminalis*", the stimulation of these endogenous stem cells may be a natural approach to reproduce the loss of neural cells during retinal degeneration. In fact, the structure of the retina at the level of *ora serrata*, specifically in its outer border known as "*ora terminalis*" may lead endogenous adult stem cells to invade from the periphery the retina. This is expected to sort beneficial effects by acting through a natural mainstream, starting from "*ora terminalis*" an area placed at the level of the so-called ciliary marginal zone where the retina switches its structures. The presence of a retinal niche of adult endogenous stem cells at this level is described in the ciliary marginal zone of the retina of zebrafish (Angileri and Gross, 2020). Such a placement of adult stem cells, despite being apparently out of place due to the extreme periphery, recapitulates indeed the placement of stem cell niches all over the CNS. In fact, the area were stem cells are mostly abundant correspond to the zone where the CNS undergoes a transition between a developed matter grey and white and the apparent atrophy which characterizes the choroid lamina. This placement corresponds to a sort of ontogenetic effort to fill what is not yet completed, during the evolution. In fact, the most active stem cell niche in the CNS corresponds to the choroid lamina of the temporal horn of the lateral ventricle. At this level, the choroid fissure exists witnessing for a yet incomplete development of the nervous mantle in the phylogenies of the human CNS. Indeed, stem cell niche seem to witness for a never-ending effort to fill in the ontogeny what the evolution did not provide yet. Thus, the apparent eccentric placement of endogenous stem cells in the eye fully overlaps with the placement of stem cell in the adult CNS. Despite the placement of stem cells in the eye is under intense research effort, their modulation to provide an effective substitutive therapy remains far to be solved. By incidence, retinal stem cells derived from differentiation of organoids possess the ACE2 receptors and were recently claimed to be a gateway to the eye for SARS-CoV-2 infection (Ahmad Mulyadi Lai, 2021). The key role of RPE is likely to be more extensive, since it is the retinal layer, which possesses at most the ability to produce intercellular signalling. At this level, as well as in the whole retina there is a marked expression of the autophagy protein LC3, which is in line with the key role of autophagy in handling protein aggregates. This occurs at first within RPE and it spreads later

to all retinal layers. Such a natural course in the needs of handling toxic cargoes throughout the various retinal layers ultimately leads to ganglionic cells as the final recipient of misfolded proteins in retinal degeneration. In fact, here is shown that these cells and mostly the M ganglionic cells, which receive the highest number of converging pathways, possess a remarkable amount of LC3. This is evident by an intense LC3 staining, which shapes the whole cell body and it is counted here by densitometry compared with other retinal layers. The occurrence of disease spreading within the CNS is related to a defect in the autophagy pathway and a similar alteration was postulated for the retinal merging of various pathological conditions (Pfeiffer et al., 2020). A defect in the autophagy machinery was even discussed as an early phenomenon in the pathogenesis of AMD (Limanaqi et al., 2020). Therefore, since the disease spreading was hypothesized to be centered on the pigment epithelium, we analysed the expression of the classic autophagy marker MAP-LC3β in baseline conditions within various retinal layers. In keeping with the hypothesis of a defective autophagy, the LC3 protein (MAP-LC3-β protein) was found to be poorly expressed within the retinal pigment epithelium and other retinal layers compared with retinal ganglion cells. In search for the seeds of the spreading of retinal degeneration here the TSG101 protein which is a component of exosomes was stained in combination with α-synuclein through various retinal layers. It is remarkable that the staining for α-synuclein quite overlaps with that of TSG101 and both are reciprocal to the pattern of TH immunostaining which is measured in the retina of pigmented mice. In fact, α-synuclein immunostaining is abundant in various retinal layers but the outer and inner nuclear layer, which corresponds to the staining pattern of the exosomal protein TSG101; at the opposite, TH immunostaining is marked and homogeneous in the outer and inner nuclear layers, while LC3 is more ubiquitous. Thus, it may be hypothesized that α-synuclein is mostly placed where synaptic activity is more intense (which rules out the nuclear layers), where the outer and inner plexiform layers are located. Similarly, the TSG101, which regulates cell-to-cell transmission is placed preferentially in the plexiform layers. This pattern confirms, in baseline conditions, the roadmap for spreading α-synuclein during retinal degeneration. In fact, in order to produce the spreading of abnormally high protein levels or aggregates of misfolded protein an effective exosomal apparatus is needed, which is witnessed by the concomitant placement of α-synuclein and TSG101. This occur where TH immunostaining is poor, which confirms a protective role for catecholamines as well as catecholamine-induced autophagy activation. Again, due to the stimulation of melanin synthesis under the effects of catecholamine activity, it is likely that the autophagy status and the key role of the RPE in clearing from protein cargoes is related to the amount of sympathetic innervation. If this true one may expect that, across retinal layers the inner and outer nuclear layers possess a higher resistance to neurodegeneration compared with the photoreceptor layer, the outer, and inner plexiform layers. Among these layers the RPE, which also possess TH innervation may play a key role to trigger or buffer the spreading of pathological processes. Similarly, the RPE in baseline conditions is expected to balance transcellular spreading of proteins across the retina.

Conclusions

in the present manuscript evidence is provided for the specific occurrence of α-synuclein in various retinal layers. This topographical pattern overlaps to that described for the exosomal marker TSG101, which suggests the presence of an apparatus which may spread α-synuclein along the retina. This may occur in baseline conditions according to the beneficial effects of the protein α-synuclein in maintaining synapse growth and cell proliferation. Nonetheless, such a system may overload the clearance activities of the retina when a degeneration affecting the autophagy pathway occurs. Since the autophagy impairment is well described in AMD and other retinal degenerations, it is likely that the widespread accumulation of α-synuclein observed in the later stages of all kinds of retinal degeneration may be due to the overwhelming of such a clearance pathway. In fact, the staining of autophagy with LC3 indicates the occurrence of such a pathway across various retinal layers to be very abundant at the final step of synaptic convergence (i.e. the great M ganglionic cells). This may explain why, at later stages of AMD,

when a non-specific retinal degeneration takes place, a multilayer α-synuclein aggregation is detected in non–specific retinal degeneration. Remarkably, a potential role of melanin under the effects of light and the stimulatory activity of the sympathetic nervous system was postulated. In fact, the melanin-inducing TH-positive sympathetic innervation was distributed in the retina according to a pattern which was complimentary to the topographical arrangement of both α-synuclein and the exosomal marker. The role of TH-positive innervation and the amount of melanin was validated by comparing the amount of TH immuno-staining in pigmented and albino rodent species. Due to a powerful protective effect of TH-positive fibers in neurodegeneration, it is likely that the absence of α-synuclein where TH activity is abundant may be not just a causal finding, but a specific casual effect. The significance of these findings extends beyond retinal layers since retinal degeneration and the role of α-synuclein and TH innervation is similarly described for neurodegenerative disorders affecting the CNS. The role of light in producing melanin-dependent toxic or protective chemical species remains to be investigated depending on the specific wave-length and the stimulation of endogenous stem cells placed at the periphery, within an area extending from *ora serrata* towards margin of the the ciliary body.

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