Melanopsin retinal ganglion cells are resistant to neurodegeneration in mitochondrial optic neuropathies

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Mitochondrial optic neuropathies, that is, Leber hereditary optic neuropathy and dominant optic atrophy, selectively affect retinal ganglion cells, causing visual loss with relatively preserved pupillary light reflex. The mammalian eye contains a light detection system based on a subset of retinal ganglion cells containing the photopigment melanopsin. These cells give origin to the retinohypothalamic tract and support the non-image-forming visual functions of the eye, which include the photoentrainment of circadian rhythms, light-induced suppression of melatonin secretion and pupillary light reflex. We studied the integrity of the retinohypothalamic tract in five patients with Leber hereditary optic neuropathy, in four with dominant optic atrophy and in nine controls by testing the light-induced suppression of nocturnal melatonin secretion. This response was maintained in optic neuropathy subjects as in controls, indicating that the retinohypothalamic tract is sufficiently preserved to drive light information detected by melanopsin retinal ganglion cells. We then investigated the histology of post-mortem eyes from two patients with Leber hereditary optic neuropathy and one case with dominant optic atrophy, compared with three age-matched controls. On these retinas, melanopsin retinal ganglion cells were characterized by immunohistochemistry and their number and distribution evaluated by a new protocol. In control retinas, we show that melanopsin retinal ganglion cells are lost with age and are more represented in the parafoveal region. In patients, we demonstrate a relative sparing of these cells compared with the massive loss of total retinal ganglion cells, even in the most affected areas of the retina. Our results demonstrate that...
Melanopsin retinal ganglion cells resist neurodegeneration due to mitochondrial dysfunction and maintain non-image-forming functions of the eye in these visually impaired patients. We also show that in normal human retinas, these cells are more concentrated around the fovea and are lost with ageing. The current results provide a plausible explanation for the preservation of pupillary light reaction despite profound visual loss in patients with mitochondrial optic neuropathy, revealing the robustness of melanopsin retinal ganglion cells to a metabolic insult and opening the question of mechanisms that might protect these cells.

Keywords: circadian rhythms; neuro-ophthalmology; mitochondrial diseases; LHON; neuropathology

Abbreviations: DOA = dominant optic atrophy; LHON = Leber hereditary optic neuropathy; mRGCs = melanopsin-containing retinal ganglion cells; RGCs = retinal ganglion cells

Introduction

Optic neuropathies are characterized by loss of retinal ganglion cells (RGCs) leading to optic atrophy and blindness. Inherited optic neuropathies due to mitochondrial dysfunction, i.e. Leber hereditary optic neuropathy (LHON) and Kjer type dominant optic atrophy (DOA), are known for initially affecting the parvocellular RGCs (papillomacular bundle), which causes dyschromatopsia, loss of visual acuity, cecocentral scotomas and temporal optic atrophy (Carelli et al., 2004). Despite loss of vision, patients with LHON and DOA are described with visual-pupillary dissociation and relatively preserved pupillary light reaction (Wakakura and Yokoe 1995; Bremner et al., 2001).

It is now known that, besides the image-forming function, the eye conveys non-image-forming light information to the brain driving the photoentrainment of circadian rhythms, masking behaviour, light-induced melatonin suppression and the pupillary light reflex (Fu et al., 2005). This system operates in parallel to the well-known image-forming pathway served by the classical outer retinal photoreceptors, the rods and cones. The non-image-forming system uses the newly identified photopigment melanopsin (Opn4) expressed in a subset of intrinsically photosensitive RGCs (Berson et al., 2002; Hattar et al., 2002). These cells project mainly to the hypothalamic suprachiasmatic nucleus, the master circadian clock, forming the so-called retinohypothalamic tract (Sadun et al., 1984; Hannibal et al., 2002, 2004; Hattar et al., 2006). They also target other areas of the brain, such as the olivary pretectal nucleus, constituting the afferent limb of the pupillary light reflex (Hannibal and Fahrenkrug, 2006; Hattar et al., 2006).

In humans, melanopsin-containing RGCs (mRGCs) comprise ~1% of the total number of RGCs, and they are located in both the RGC layer and the inner nuclear layer (Hannibal et al., 2004; Dacey et al., 2005). These mRGCs have large dendritic fields, which run predominantly in the outer sublayer of the inner plexiform layer. The dendrites contain melanopsin and respond to light by depolarization. The mRGCs also receive input from rods and cones through the bipolar and amacrine cells, which modulate their activity (Viney et al., 2007). There is substantial evidence that melanopsin is a photopigment exhibiting bistability (Mure et al., 2007), which uses an invertebrate (rhabdomeric) signal transduction pathway (Peirson and Foster, 2006). Melanopsin-containing RGCs are now considered the ‘circadian photoreceptors’ and their function explains the maintenance of photoentrainment of circadian rhythms in mouse models and human ocular diseases characterized by loss of rods and cones (Foster et al., 1991; Freedman et al., 1999; Hannibal et al., 2004). Furthermore, the preservation of light-induced melatonin suppression has been demonstrated in blind subjects, including patients with various optic neuropathies (Czeisler et al., 1995; Hatanen et al., 1998; Perez-Rico et al., 2009).

The present study was designed to investigate whether, in mitochondrial optic neuropathies such as LHON or DOA, mRGCs are affected to an extent that would produce circadian rhythm disturbances. Furthermore, we describe the characteristics and distribution of mRGCs in normal human retina and in hereditary optic neuropathies.

Materials and methods

Melatonin suppression test by light

We studied nine control subjects (mean age 34.33 ± 8.96) and nine subjects with molecularly confirmed mitochondrial optic neuropathy, five LHON and four DOA (mean age 35.33 ± 9.89). The study protocol was approved by the Internal Review Board ethical committee and written informed consent was obtained from both controls and patients. All the subjects enrolled in the study reported no history of working night shifts nor did they travel more than two time zones within 1 month prior to the test. The subjects had not taken drugs interfering with nocturnal melatonin synthesis (i.e. beta-blockers, alpha-blockers, non-steroidal anti-inflammatory drugs, serotonin reuptake inhibitors, neuroleptics, antiepileptics and monoaminooxidase inhibitors). All control subjects had normal vision. The hereditary optic neuropathy subjects had an extensive ophthalmologic evaluation including, as an objective indicator of their clinical severity, optical coherence tomography (StratusOCT, software version 3.0; Carl Zeiss Meditec Inc., Dublin, CA) measurements of retinal nerve fibre layer thickness (RNFL 3.4 protocol). To characterize the chronotype, psychiatric disturbances, sleep disturbances and quality of life, all subjects filled out self-administered questionnaires (Horne-Östberg Morningness–Eveningness Scale; Pittsburgh Sleep quality index, Zung depression and anxiety scales, SF-36 quality of life scale, Epworth Sleepiness Scale and Berlin Questionnaire). This study was carried out between December 2007 and May 2008.

The study protocol consisted of one baseline night and one light exposure night (Supplementary Fig. 1). A sleep diary was used the week prior to the test to monitor the sleep-wake cycle. Subjects were asked to refrain from alcohol 48 h prior to each test and not drink coffee or eat bananas, cheese or chocolate 12 h before the
test. For the baseline night, all subjects were lying down in the bed in a dim light (<5 lux) room and were blindfolded from 10 pm to 8 am. The same setting was used for the melatonin suppression night except for the exposure to monochromatic (470 nm) blue light from 1:30 to 3:30 am (Supplementary Fig. 1). The light was generated by two vertical lamps (38 LEDs) that projected light onto the ceiling (Sivra, Guzzini\(^\text{\textregistered}\), Italy). The ceiling surface that reflected this light was 100 cm × 150 cm and positioned ~2 m from the subject eyes (Supplementary Fig. 1). Irradiance at the level of the subjects’ corneas was 58 μW/cm\(^2\), as detected by spectroradiometric assessment. Blood samples were collected each hour from 00:30 to 3:30 am and melatonin plasma concentrations evaluated by radioimmunoassay (Melatonin Direct RIA-KIPL3300; Pantec-Biosource Europe S.A.). The minimum detection limit of the assay was 2 pg/ml. The magnitude of melatonin suppression was assessed by two previously reported control-adjusted scores: melatonin suppression score and suppression rate (Brainard et al., 2001; Hebert et al., 2002). We also computed the percentage difference between melatonin plasma levels before and after light exposure in the melatonin suppression night (absolute percentage difference) (for details see Supplementary material).

**Immunohistochemical analysis of melanopsin-containing retinal ganglion cells in human retinas**

Eyes with optic nerves were obtained post-mortem from two male subjects with LHON (59- and 52-year-old) and from one male subject with DOA (87-year-old). Eyes were also acquired from an eye tissue bank (Lions Eye Bank of Oregon, USA) for age- and sex-matched controls (males; 58, 54 and 85 years old, respectively). We excluded tissues from subjects with a history of diabetes and neurological disorders. All tissues were initially immersion-fixed in neutral buffered formalin. Eyes and nerves were oriented for superior and temporal zones with tissue ink. The eyes were dissected horizontally at the meridian producing two colottes containing the entire retina (nasal and temporal), at that level bisecting the papillomacular bundle. Tissue from the superior half was embedded in paraffin and serially sectioned at 5 μm. Sections were immunostained for melanopsin (rabbit anti-melanopsin, code no. 5168, characterized in detail by Hannibal et al. (2004)) using an indirect immunoperoxidase technique with diaminobenzidine as the substrate/chromogen or immunofluorescence (Hannibal et al., 2004). Immunostaining was performed on seven to ten serial sections to define the extent of each single mRGC and establish the counting criteria for subsequent quantitative analysis. To this end, mRGCs were then identified and manually counted by two independent observers on five to six sequential slides originating from every fifth section. Melanopsin stained RGCs were further confirmed and photographed at high magnification by the two observers. We counted each mRGC that demonstrated a significant amount of stain indicating a complete soma and having the nucleus and sometimes the nucleolus visible. In these latter cases, the cells were considered to be cut approximately through their middle. Furthermore, the length of each retina section was measured on serial photographs overlapping on the borders, covering the entire retina available. Counting of mRGCs allowed for calculation of their area density across the posterior retina in the nasal/temporal axis. This retinal cross-section, 5 μm in thickness, included cells cut approximately through their middle, with the nucleus visible. Hence, the sampling area around each section would be large, 2-fold (one for each side) the mean radius of the mRGC (5 μm) plus the thickness of the retinal section (5 μm), so that the density has been calculated dividing the number of mRGCs for the total sampling area (length of the retinal cross-sections multiplied by 15 μm). The total retinal area density of mRGCs in each eye was calculated assuming an average total surface in human retina of 1040 mm\(^2\) and an even superior/inferior distribution of mRGCs (Curcio and Allen, 1990).

Total RGC numbers were calculated through measurements in the optic nerve and based on the assumption that one RGC produces one axon that passes through the optic nerve. Optic nerve cross-sections were previously analysed by axonal morphometry for normal subjects at different ages with paraphenylenediamine-based image analysis (Tenhula et al., 1992). Thus, to obtain the ratio of mRGCs on the total number of RGCs for the control eyes, we considered an average of 1 200 000 RGCs per retina, as previously reported by our laboratory (Johnson et al., 1987). For the two LHON subjects, the total number of mRGCs was measured by counting the axons in the optic nerve cross-sections, using similar paraphenylenediamine-based manual count (see next section). For the DOA subject, the counting of axons in the only optic nerve cross-section available was performed by identifying axons using an immunostaining for neurofilaments in sections cut from paraffin-embedded blocks, not having plastic-embedded tissue available.

Each retinal section was divided into two hemiretinans (nasal and temporal) assuming the macula as the zero point centred 15° temporal to the optic disc. Position of each mRGC, either in the inner nuclear layer or in the RGC layer, was reported in grades considering its distance from zero on a proportional scale in which the maximum length was 60° and 90° for temporal and nasal hemiretina, respectively.

For statistical analysis (see below), we also calculated the number of mRGCs in each 20° sector, starting from the parafoveal sector (10° nasally and 10° temporally to the macula).

**Morphometric assessment in optic nerves**

Optic nerves were cut into cross-sections 2 mm thick and ~3 mm posterior to the globe. Orientation was established by razor nicks and the specimens were processed for paraffin and plastic blocks. The paraffin tissue blocks (DOA subject) were cut with a microtome at 5 μm, stained with haematoxylin and eosin and immunostained using an indirect method with horseradish peroxidase and diaminobenzidine as substrate/chromogen for neurofilaments. Semi-thin sections were cut on an ultramicrotome at 1 μm from plastic-embedded tissues (LHON subjects) and stained with paraphenylenediamine for light microscopic examination of myelin profiles. All light microscopic photos of eyes and nerves were acquired with a Spot II digital camera (Diagnostic Instruments Inc.) and digitally saved on a computer.

The axonal count in the optic nerves from the two LHON and DOA subjects was manually performed on images acquired at 1000 × with a 100 × oil immersion lens. The optic nerve cross-section profiles were divided into five regions, each with a different axonal density, to account for the non-homogeneous distribution of axonal loss (for details on the sampling method applied, see Methods section in Supplementary material). Each image was reviewed by two independent observers. The counts for the five regions were summed to obtain the total axon count for each optic nerve.

**Statistical analysis**

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS ver.12.0, Chicago, IL). Light-induced suppression of
melatonin secretion was evaluated by means of a paired-sample t-test comparing plasma melatonin levels at the corresponding time point in the baseline and light suppression nights. Differences between the groups in melatonin plasma levels and optical coherence tomography measurements were assessed by one-way ANOVA considering significant a P-value lower than 0.05. For self-administered questionnaires, a non-parametric analysis (Kruskal–Wallis) was performed. Correlation between melatonin suppression scores and clinical variables was assessed by using Pearson coefficient for questionnaires and Spearman analysis for ophthalmologic data. To evaluate the homogeneity of mRGC distribution throughout the retina, we performed a one-way ANOVA, comparing their mean number between different 20° retina sectors for each group (see Figs 2 and 4 as well as Supplementary material). Moreover, by means of parametric statistic, we compared the number of mRGCs in controls and optic neuropathy subjects, both in the entire retina and for each equivalent sector.

## Results

### Melatonin suppression test by light

Exposure to light during night suppresses melatonin secretion (Klein and Weller, 1972) and this response is conveyed by the mRGC–retinohypothalamic tract pathway (Brainard et al., 2001). A few studies investigated this response in blind human subjects showing that it may be preserved (Czeisler et al., 1995; Hatonen et al., 1998; Perez-Rico et al., 2009). However, the blind subjects investigated were not homogeneous for pathology and only a few were affected by optic neuropathy of variable aetiology. We aimed to explore the light-induced suppression of melatonin secretion in a homogeneous group of nine patients affected by mitochondrial optic neuropathy (five LHON and four DOA), which selectively affects the RGCs, compared with nine sex- and age-matched controls. Demographic, genetic and ophthalmologic data of patients with optic neuropathy are provided in Table 1. The results of the melatonin suppression tests for controls and optic neuropathy subjects are shown in Fig. 1.

All optic neuropathy subjects had poor vision but a relatively preserved pupillary light reflex. Mean visual acuity and standard deviation of the better eye was 0.09 ± 0.1 for LHON subjects and 0.2 ± 0.1 for DOA subjects, with no significant differences. Thus, the two mitochondrial optic neuropahties were grouped together for further analysis. All optic neuropathy subjects showed severe optic atrophy on fundus examination. Optical coherence tomography evaluation revealed a substantial reduction of retinal nerve fibre layer thickness in both optic neuropathies (Fig. 1A and Supplementary Table 1). Self-administered questionnaires assessing quality of life, sleep and mood disturbances did not reveal significant differences between controls and optic neuropathy subjects except for items A (physical functioning) and C (bodily pain) of Short Form 36-Item quality of life scale (raw data for controls and optic neuropathy subjects are provided in Supplementary Tables 2 and 3).

The study design, described in the Methods section, included a basal night and a test night (Supplementary Fig. 1). All subjects showed a physiological increase of melatonin plasma levels during the baseline night, but the melatonin plasma levels were higher (P < 0.05) in optic neuropathy subjects than controls (Fig. 1B). A significant suppression of melatonin plasma levels induced by blue light was observed in both control (P < 0.0001) and optic neuropathy subjects (P < 0.005) (Fig. 1B). Individual profiles of baseline and light suppression nights are provided in Supplementary Figs 2 and 3. Furthermore, we compared the results of the melatonin suppression test in LHON and DOA subjects and found no significant differences (data not shown). Specifically, the magnitude of suppression was not statistically different between groups, for each of the three indices considered: control adjusted melatonin suppression score (P = 0.9), suppression rate (P = 0.5) and absolute percentage difference (P = 0.64) (Fig. 1C). We failed to identify any correlation between melatonin suppression scores and the ophthalmologic outcome measurements (i.e. retinal nerve fibre layer thickness by optical coherence tomography and visual acuity). A positive correlation was detected only between the absolute percentage difference score and the Pittsburgh sleep quality index (P = 0.019) in both controls and optic neuropathy subjects.

These results suggest that light suppression of melatonin secretion is maintained in visually impaired subjects with mitochondrial optic neuropathy, indicating that the mRGC-retinohypothalamic tract pathway is essentially spared. This observation is in agreement with the relative preservation of the pupillary light reflex, as assessed at clinical exam in the patients with LHON and DOA.

### Table 1 Demographic, genetic and ophthalmologic data

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<th>ID</th>
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<th>Mutation</th>
<th>Onset</th>
<th>Visual acuity</th>
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<td></td>
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<tr>
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<td>11778/ND4 mtDNA (homoplasmic)</td>
<td>15</td>
<td>0.01</td>
</tr>
<tr>
<td>L3</td>
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<td>3460/ND1 mtDNA (homoplasmic)</td>
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<tr>
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<td>M</td>
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<td>14484/ND6 mtDNA (heteroplasmic)</td>
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<tr>
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<tr>
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<tr>
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Characterization of melanopsin-containing retinal ganglion cells in human retinas

Despite the plethora of histological studies investigating mRGCs in different animal species, only a few have characterized this cellular system in human retinas (Hannibal et al., 2004; Dacey et al., 2005; Vugler et al., 2007). To fill this gap, we investigated three pairs of eyes from normal donor subjects: a 58-year-old (Control 1), a 54-year-old (Control 2) and an 85-year-old (Control 3) male subject. The oldest subject was investigated to take into account the ageing process; in fact, previous studies from our group showed that after the age of 60 there is a progressive loss of axons that corresponds to a decrease of RGCs (Johnson et al., 1987).

Immunohistochemistry, using human melanopsin antibodies on sagittal sections of the retina, revealed the presence of mRGCs and their dendrites with the cell bodies either in the RGC layer...
or in the inner nuclear layer, as previously reported (Fig. 2A) (Hannibal et al., 2004). The mRGCs were characterized by having a large cell body (15–20 μm) with a centrally located nucleus. The pigment was located primarily in the membrane of the soma and in the dendrites. The dendrites were located in two different layers. The majority of dendrites were found at the border of the inner nuclear layer known as the OFF layer of the inner plexiform layer. The dendrites were also located close to RGC layer, known as the ON layer of the inner plexiform layer. Occasionally, delicate thin axons were also identifiable by their melanopsin brown staining as they ran from the RGC soma into and along the retinal nerve fibre layer (Fig. 2A).

To evaluate the ratio of mRGCs relative to the total number of RGCs in human retinas, we assumed that the number of RGCs is equivalent to the number of axons counted in optic nerve cross-sections. Thus, we used 1200,000 as the average number of RGCs in controls younger than 60 years of age, as reflected by optic nerve axonal counts previously carried out in our laboratory (Tenhula et al., 1992). To evaluate the loss of axons with ageing, we manually counted them in an optic nerve cross-section from the 85-year-old subject, obtaining an estimate of 978,000 axons. To obtain the mRGC density relative to retinal surface, we assumed that the mRGCs are homogeneous in distribution over the 1040 mm² established total retinal surface (Curcio and Allen, 1990). Thus, quantitative evaluation by manual counting of mRGCs resulted in a mean density of ~18 cells/mm² for Control 1 and 13 cells/mm² for Control 2, whereas the mean density was 8 cells/mm² for Control 3 (Supplementary Table 4). Finally, the percentage of mRGCs, over the total number of RGCs, was 1.5% for Control 1, 1.1% for Control 2 and 0.9% for the oldest, Control 3 (Fig. 2B). We next evaluated how many of the mRGCs were located in the RGC layer and in the inner nuclear layer, obtaining comparable numbers in the two layers (Supplementary Fig. 4 and Table 4). Overall, these data indicate that in humans, the number of mRGCs in the RGC layer is about equal to those located in the inner nuclear layer, at odds with the previous estimations in rodents (Bauer et al., 2008).

A further objective of our study was to describe the distribution of mRGCs on the two hemiretinas, nasal and temporal, relative to the fovea (central retina) by using intervals of 20° (for reconstruction of the retina, see the ‘Methods’ section and Supplementary Fig. 5). The 20° range was chosen in order to have a larger sample size per angle and for isolated comparison of the parafoveal and peripapillary zones. The overall estimation for the two younger controls showed a higher number of mRGCs in the parafoveal region centred 15° temporal to the optic disc (Fig. 2C, left panel), which was close to significance when compared with the far temporal end (P = 0.052). A second enrichment in mRGCs was observed at the far end of the nasal hemiretina. Comparing these results with those obtained in the oldest subject, we observed a general loss of mRGCs in the latter except for those in the parafoveal region (Fig. 2C, right panel), which was significantly different from the remaining retinal sectors (P = 0.05) except for the far nasal end. Thus, these results indicate a general loss of mRGCs that is age related, which spares the parafoveal region. This overall decrease of mRGCs in the oldest subject parallels the previously described reduction of axons (and RGCs) after the age of 60 in humans (Johnson et al., 1987) and is similar to previous descriptions of mRGC loss with ageing in animals (Semo et al., 2003).

Our characterization of mRGCs in normal and ageing humans showed that these cells constitute ~1% of total RGCs, are roughly equally distributed in the RGC layer and the inner nuclear layer and are more represented in the central retina. The mRGCs, like other RGCs, become less numerous with age. These results corroborate previous reports using flat-mounted retinas (Hannibal et al., 2004; Dacey et al., 2005), validating our methodology of using serial retinal sections sagittal to the optic nerve head and with temporal–nasal orientation.

**Analysis of melanopsin-containing retinal ganglion cells in mitochondrial optic neuropathy patients**

To explore directly the histopathology of mRGCs in mitochondrial optic neuropathies, we studied retinal specimens collected at autopsy from patients with LHON and DOA, comparing them with control subjects. We had available four eyes from two LHON subjects, one eye from a DOA individual, and the above described six eyes from three age- and sex-matched controls for comparison.

The two LHON subjects were brothers, included in a prospective clinical study, thus systematically evaluated prior to death (Sadun et al., 2003). They both belonged to a large Brazilian pedigree of Italian maternal ancestry carrying the 11778/ND4 mutation in mitochondrial DNA (Carelli et al., 2006). However, the severity of optic neuropathy differed markedly in these two subjects. The 59-year-old man had a late-onset and mild form of LHON, whereas his 52-year-old brother had a classical LHON with young–adult onset and severe optic atrophy. The neuro-ophthalmologic evaluation of these two LHON subjects is summarized in Table 2 and Figs 3A and B.

The patient with mild LHON suffered visual loss at 51 years of age precipitated by abuse of tobacco and alcohol; he remained mildly affected until his death. His fundus examination revealed mild optic neuropathy with temporal optic atrophy (Fig. 3A, upper panels). Post-mortem evaluation of the optic nerves revealed a similar sectoral loss of axons (Fig. 3A, middle panels). Examination at higher magnification documented the remaining axonal density as high nasally, intermediate in the transition zone and very low temporally (Fig. 3A, lower panels).

The patient with severe LHON suffered visual loss at 27 years of age and remained stable for 25 years before he died. Fundus examination showed complete optic atrophy bilaterally (Fig. 3B, upper panels). Post-mortem evaluation of the optic nerves revealed reduced cross-sectional profiles with massive axonal loss throughout except for some sparing nasally (Fig. 3B, middle panels). Examination at higher magnification documented the remaining axonal density as depleted nasally, low in the transition zone and very low temporally (Fig. 3B, lower panels).

The patient with DOA was an 87-year-old man, belonging to a family of 152 members reported by Kjer in the seminal description of DOA (Kjer, 1959). The frame-shift inducing 2826delT (p.V942fsX967) mutation in exon 28 of the OPA1 gene was
Figure 2  (A) Melanopsin retinal ganglion cells in control retinas. Upper line: (left) one example of a brown-stained mRGC located in the RGC layer is shown including the large cell body with the nucleus and a dendrite running at the border of the inner nuclear layer; (middle) a brown-stained mRGC located in the inner nuclear layer is depicted; (right) the peripheral staining of melanopsin under the plasma membrane is evident in this mRGC located in the RGC layer. Lower line: (left) one example of a long dendrite running close to the RGC layer is shown; (right) one example of a thin axon filled with brown-stained melanopsin in the retinal nerve fibre layer is depicted (scale bar represents 20 μm).  

(B) Ratio of mRGCs relative to the total number of RGCs in control retinas. The ratio of mRGCs relative to the total number of RGCs is shown for Control 1 (left), Control 2 (middle) and Control 3 (right), respectively.  

(C) Retinal distribution of mRGCs in control subjects. Distributions of mRGCs in four eyes from averaged Controls 1 and 2 (left) and in the two eyes from Control 3 (right) are shown. The mean number of mRGCs (± SD) is reported for each 20° sector of the temporal and nasal hemiretinas, centred to the fovea. Mean number of mRGCs in parafoveal sector (10°−n10°) is greater in the two younger controls, being close to statistical significance with respect to the far temporal hemiretina (P = 0.052). In the oldest, Control 3, the number of mRGCs in parafoveal sector (f10°−n10°) is also greater and significantly different (P = 0.05) with respect to all sectors except for n50°−n70° and n70°−n90° (f10°−n10° versus: f60°−f50° P = 0.01; f50°−f30° P = 0.024; f30°−f10° P = 0.024; f10°−n30° P = 0.024; n30°−n50° P = 0.015). RNFL = retinal nerve fibre layer; RGCL = retinal ganglion cell layer; IPL = inner plexiform layer; INL = inner nuclear layer.
recently described (Thiselton et al., 2002). This patient complained of decreased vision and visual difficulties in poor light and in colour perception. His visual acuity was 0.1 in oculus destrum (right eye) and counting fingers in oculus sinistrum (left eye). Fundus examination revealed diffuse optic atrophy (Fig. 3C). The histopathology of this subject’s eyes has been previously reported (Kjer et al., 1983). We had available the left optic nerve for this study, and a severe loss of axons affected most of the cross-section, similar to the findings in the LHON subjects (Fig. 3C).

Immunohistochemistry with melanopsin antibodies on the retinas from these LHON and DOA subjects revealed the presence of mRGCs and of their dendrites similar to the control subjects, notwithstanding the severe loss of total RGCs (Fig. 4A). In terms of gross morphology, dimension and dendritic branching pattern, these mRGCs did not differ from those described in the control retinas.

The residual axons were counted manually in the optic nerve cross-sections from the optic neuropathy cases. This count revealed an average between the two eyes of \( \frac{300,000}{2} \) axons for the mild LHON (74% loss as compared with 1,200,000 axons in normal eyes) and \( \frac{22,500}{2} \) for the severe LHON (98% loss) and 15,000 for the DOA (94% loss as compared with 977,851 axons counted for the age-matched control eye) (Fig. 4B, left panel). Quantitative evaluation by manual counting of mRGCs resulted in a density of 9 cells/mm\(^2\) for mild LHON, 8 cells/mm\(^2\) for severe LHON and 7 cells/mm\(^2\) for the DOA subject (Supplementary Table 4). These counts were approximately half of those obtained for the two younger controls (Fig. 4B, middle panel).

For LHON subjects, the total RGC number was based on direct axonal counts in the corresponding optic nerve cross-sections, as described above. Thus, the percentage of mRGCs was increased as the total number of RGCs decreased. This percentage was 3.1% for mild LHON and 38.5% for severe LHON compared with an average of 1.3% for the two age-matched controls (Fig. 4B, right panel). To match the age of the DOA subject we used the 85-year-old Control 3. Thus, the percentage of mRGCs, compared with the total number of RGCs, was 48.6% for the DOA subject and 0.9% for Control 3 (Fig. 4B, right panel). The mRGCs were approximately equally distributed in the RGC layer and inner nuclear layer in the optic neuropathy cases, similar to control subjects, indicating that the relative distribution of mRGCs in RGC layer and inner nuclear layer among both control and optic neuropathy subjects was not different (see Supplementary Fig. 4 and Table 4).

The analysis of mRGC distribution for nasal and temporal hemiretinas in the two LHON subjects shows that despite an overall absolute loss of mRGCs, more remain in the parafoveal region (Fig. 4C, left panel). This result was significantly different from sectors \( 60^\circ - 150^\circ \), \( 150^\circ - 130^\circ \) and \( 50^\circ - 70^\circ \) (\( P = 0.002 \), 0.04 and 0.008, respectively). The retina from the patient with DOA had an essentially uniform distribution of mRGCs, not showing the parafoveal enrichment (Fig. 4C, right panel). A sector-by-sector comparison of the optic neuropathy subjects with the corresponding age-matched controls failed to reveal significant differences.

Table 2: Demographic, genetic and ophthalmologic findings in mild LHON, severe optic atrophy and DOA subjects

<table>
<thead>
<tr>
<th>ID</th>
<th>Sex</th>
<th>Age</th>
<th>Mutation</th>
<th>Onset</th>
<th>Visual acuity</th>
<th>Pupillary light reflex</th>
<th>Visual fields</th>
<th>Fundus</th>
<th>Scanning laser polarimetry</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>59</td>
<td>11778/ND4 mtDNA (homoplasmic)</td>
<td>51</td>
<td>Moderately decreased</td>
<td>Moderate collapse</td>
<td>Temporal atrophy</td>
<td>Normal</td>
<td>OD: Temporal atrophy, OS: Central scotoma</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>52</td>
<td>11778/ND4 mtDNA (homoplasmic)</td>
<td>27</td>
<td>Severe collapse</td>
<td>Complete collapse</td>
<td>Complete optic atrophy</td>
<td>Normal</td>
<td>OD: Complete optic atrophy, OS: Central scotoma</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>84</td>
<td>Mut. OPA1 Exon 28; 2826delT (p.V942fsX967)</td>
<td>Childhood</td>
<td>0.1 Counting fingers</td>
<td>Normal</td>
<td>Normal</td>
<td>OD: Normal, OS: Central scotoma</td>
<td></td>
</tr>
</tbody>
</table>

n.a. = not available; OD = oculus destrum; OS = oculus sinistrum.
The overall comparison of all 11 eyes from controls and optic neuropathy subjects investigated is depicted in Fig. 4D. This demonstrates clearly the non-uniform distribution of mRGCs in human retinas, with a significant enrichment in the parafoveal region that is maintained despite neurodegeneration in LHON and ageing. The single retina from the DOA case, exposed to the combination of both neurodegeneration and ageing, showed a flat distribution of mRGCs.

Discussion

We investigated a class of optic neuropathies characterized by selective and massive loss of RGCs and preservation of the pupillary light reflex (Wakakura and Yokoe 1995; Bremner et al., 2001; Carelli et al., 2004). In these optic neuropathies, RGC loss is due to a neurodegenerative process caused by mitochondrial dysfunction, and in the present study we document the resistance of mRGCs to this pathogenic mechanism. Nine patients with LHON or DOA maintained light-induced suppression of melatonin nocturnal secretion comparable to nine control subjects, despite their severe visual loss and optic atrophy. We examined 11 retinas from optic neuropathy subjects and age-/sex-matched controls and we demonstrated the relative preservation of mRGCs compared with total RGC loss in affected subjects. Furthermore, we characterized the mRGC system in human retinas, showing that these photoreceptors are more concentrated around the macula and undergo a reduction in numbers with ageing, possibly...
Figure 4 (A) Melanopsin cells in control and optic neuropathy retinas. In the upper line, examples of brown-stained mRGCs located both in the RGC layer and in the inner nuclear layer for all three controls are provided (scale bar represents 60 μm). The Control 3 sections were stained by immunofluorescence and all nuclei are red, whereas melanopsin is green (scale bar represents 50 μm). Single to multilayered RGCs are shown in these pictures. In the lower line, examples of brown-stained mRGCs located in the RGC layer in all three optic
explaining the higher incidence of circadian rhythm disorders, such as sleep disturbances in elderly persons. The current results provide a plausible explanation for the relatively preserved pupillary light reaction despite profound visual loss in these patients, revealing the robustness of mRGCs to a metabolic insult and opening the question of mechanisms that might protect these cells.

Our study stems from a few reports in the literature showing that patients with optic neuropathy may maintain the light-induced melatonin suppression response at night, including two DOA subjects (Czeisler et al., 1995; Hatonen et al., 1998; Perez-Rico et al., 2009). In contradistinction, a study of patients with various optic neuropathies reported the frequent occurrence of sleep timing disorders, which may indicate a defective photo-entrainment of circadian rhythms (Wee and Van Gelder, 2004). In our study, the mitochondrial optic neuropathy subjects, who were homogeneous for pathology, had no differences compared with controls in terms of melatonin suppression even if with larger variability. Remarkably, the maintenance of light-induced melatonin suppression was unrelated to the severity of the optic neuropathy in these patients, strongly suggesting that despite the dramatic loss of total RGCs, the mRGCs were still able to convey light information to the pineal gland through the retinohypothalamic tract. It is worth noting that optic neuropathy subjects showed higher melatonin levels during the baseline night than controls; this may indicate a certain rearrangement of melatonin secretion induced by changes in the pattern of light input because of RGC loss (Lubkin et al., 2002).

To corroborate these results, we studied post-mortem human retinas from patients with optic neuropathy and controls. We first characterized the mRGC system in normal human retinas using sagittal sections from standard paraffin embedded eyes, having validated a protocol for cell counting in serial sections stained with melanopsin antibodies. Using this approach, we reached results strikingly similar to previous estimates of mRGCs in flat-mounted human retinas, showing that in middle-aged normal people mRGCs represent ~1% of total RGCs, approximately equally distributed in RGC layer and inner nuclear layer (Hannibal et al., 2004). Furthermore, we report here the first evaluation of aging on mRGCs in humans, demonstrating that after age of 80 there is a reduction of mRGCs, which is about the same rate as the general reduction of RGCs. This observation is concordant with previous reports on the age-related decrease of mRGCs in animal models (Semo et al., 2003). A further finding of our study was that mRGCs are more concentrated in the parafoveal region, as previously reported (Dacey et al., 2005; Jusuf et al., 2007). A second peak of mRGC numbers was observed at the far end of the nasal hemiretina, which was not previously documented in human retinas. It is tempting to speculate that for non-image-forming and time-integrated functions, the far nasal retina is well positioned to see the temporal peripheral field, where the background illumination can be observed rather than the foreground. However, in animals, different patterns of mRGC distribution have been found (Hattar et al., 2002; Semo et al., 2005).

The analysis of pathological retinas from patients with mitochondrial optic neuropathy revealed a relative sparing of mRGCs, as shown by the increase of their ratio over total RGCs from ~1% in controls up to 49% in the most severe case. These cells were clearly present in the retinal regions most severely depleted by neurodegeneration, such as the parafoveal area, which generates the papillomacular bundle (Sadun et al., 2000). In terms of absolute numbers, mRGCs are reduced to about half in the LHON cases compared with controls. In the case of the DOA subject the absolute number of mRGCs was about equal to the control. This may be explained by the age-related reduction of mRGCs seen in combination with the neurodegenerative cell loss in the DOA case.

Our results show that mRGCs are lost in patients with LHON and DOA but at a much slower rate compared with the regular RGCs. In fact, this loss does not result in a defective light-induced suppression of melatonin secretion, which indicates a sufficient preservation of the retinohypothalamic tract. This observation in living patients is consistent with a recently published study, which reported that it requires ~80% loss of mRGCs to manifest

**Figure 4 Continued**

neuropathy subjects are shown (scale bar represents 60 μm). Their persistence is remarkable despite the complete absence of the other RGCs. The DOA sections were stained by immunofluorescence and all nuclei are red, whereas melanopsin is green (scale bar represents 25 μm). (B) Axonal (RGCs) and mRGC count, and mRGC/RGC ratio for control, LHON and DOA subjects. On left, histograms of axonal counts for averaged Controls 1 and 2, mild LHON (mLHON), severe LHON (sLHON), Control 3 and DOA subjects are shown demonstrating a severe loss of axons in optic neuropathy subjects. In the middle, histograms of mRGC counts for averaged Controls 1 and 2, mild LHON, severe LHON, Control 3 and DOA subjects are shown revealing a relative preservation of mRGCs in the optic neuropathy subjects as compared with the severe rate of axonal loss, which equals total RGCs. On the right, the ratio of mRGCs relative to the total number of RGCs is shown for averaged Controls 1 and 2, and Control 3 (upper line), and for mild LHON, severe LHON and DOA subjects (lower line). In the optic neuropathy subjects there is a striking increase in the rate of mRGCs, which is inversely correlated with the severity of RGC loss, indicating their relative preservation. (C) Retinal distribution of mRGCs in subjects with optic neuropathy. Distributions of mRGCs in four eyes from averaged mild LHON and severe LHON (left) and one eye from DOA (right) are shown. The mean number of mRGCs (±SD) is reported for each 20° sector of temporal and nasal hemiretinæ, centred on the fovea. Mean number of mRGCs in parafoveal sector (110°–110°) is greater in the two LHON subjects, being significantly different compared with the sectors 160°–150°, 50°–10° and 50°–70° (P = 0.002, 0.04 and 0.008, respectively). In the DOA case the number of mRGCs is uniformly distributed, lacking a parafoveal peak. (D) Overall retinal distribution of mRGCs in control and optic neuropathy subjects. The distribution of mRGCs for each eye of Controls 1–3 and for mild LHON, severe LHON and DOA cases are shown. The relative size of the black circles represent the number of mRGCs for each 5° sector (the x-axis shows the fovea as point 0 and the other sectors as relative to the centre, being negative for the temporal hemiretina and positive for the nasal hemiretina). In the last line the cumulative distribution of mRGCs is reported for all controls, all optic neuropathy subjects and merged for everybody. OD = oculus destrum; OS = oculus sinistrum.
abnormal non-image-forming visual responses in a mouse model with targeted destruction of mRGCs (Goz et al., 2008). Similarly, the observed sparing of mRGCs would explain the relative maintenance of pupillary light reflex despite profound visual impairment in LHON and DOA (Bremner et al., 1999, 2001). We have previously shown, in a post-mortem study of a single LHON case with an extremely severe optic atrophy (Sadun et al., 2000), spared axons leaving the optic chiasm and projecting to the pretectum that constitute the afferent arm of the pupillary light reflex (Bose et al., 2005). Furthermore, we also showed, using selective wavelength pupillometry, that the pupil contraction amplitude to blue light, which specifically stimulates mRGCs, was minimally reduced in the only affected eye in a unilateral case of LHON (Kawasaki et al., 2010).

Although the current study is the most extensive in human subjects to date, the number of investigated cases is still limited, especially the post-mortem histopathology of DOA (Kjer et al., 1983). Thus, for DOA our conclusions are not as firm as they are for LHON. Indeed, an abnormal masking response on the circadian running wheel was reported in an OPA1 mouse model of DOA (Davies et al., 2007). However, the testing paradigm and the limited loss of RGCs found in this mouse make comparison between our results and these observations difficult. Our results on the single DOA case show partial sparing of mRGCs similar to LHON subjects, yet further investigations are needed to resolve these issues.

The present study reveals a robustness of mRGCs despite mitochondrial dysfunction and leads to the question of which mechanisms underlie this resistance to neurodegeneration. There is a previous body of evidence that supports the current findings. Remarkably, an old study of RGC loss after optic nerve transaction showed that a small subset (~1%) of cells with large bodies, intensively stained with cytochrome c oxidase (complex IV), survived retrograde cell death (von Bussmann et al., 1993). It is tempting to speculate that these spared cells, with high mitochondrial activity as shown by the intense cytochrome c oxidase staining, were mRGCs. In fact, mRGCs were shown to be partially spared in a more recent experimental model of axotomy (Robinson and Madison, 2004). Studies of mRGCs in animal models of glaucoma with high intraocular pressure have also shown mRGC survival (Li et al., 2006). A further evidence of mRGC robustness comes from studies on cell toxicity to monosodium glutamate, showing that despite severe loss of RGCs, the mRGCs remain and are functional (Chambille and Serviere, 1993; Hannibal et al., 2001).

We do not presently know if mRGCs present distinctive features in mitochondrial metabolism compared with the regular RGCs. The latter are obviously vulnerable to mitochondrial dysfunction, being the selective target for cell death in mitochondrial optic neuropathies such as LHON and DOA. RGCs have axons that run an unmymelinated and long stretch in the intraocular retinal nerve fibre layer. This absence of myelin, necessary for optical transparency, also imposes a very high energy requirement upon the RGCs (Carelli et al., 2004). Another hypothesis is that light exposure to the RGCs and their axons may be directly harmful, especially in the setting of dysfunctional mitochondria, as in these optic neuropathies (Osborne et al., 2008). It is intriguing to speculate that mRGCs may be intrinsically protected from light damage by expressing melanopsin photopigment. Specific investigations are needed to explore the neuroprotective mechanisms that may preserve mRGCs.

In conclusion, two important implications for medicine are drawn from our study. Firstly, notwithstanding their low vision, patients with LHON and DOA have retained the anatomical circuit supporting the light entrainment of circadian rhythms, thus avoiding consequences of circadian misalignment. Second, elucidating the distinguishing features of mRGC mitochondrial metabolism will improve our understanding of intimate pathogenic mechanisms and provide novel approaches for therapeutic interventions in these currently untreatable inherited optic neuropathies.

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Supplementary material

Supplementary material is available at Brain online.

References


