Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Scienze Mediche Specialistiche
PFDR in Medicina del Sonno

Ciclo XXIV

Settore Concorsuale di afferenza: 06/D6 Neurologia
Settore Scientifico disciplinare: MED 26

Melanopsin Retinal Ganglion Cells: relevance to circadian rhythms and sleep in neurodegeneration

Presentata da: Dott.ssa Chiara La Morgia

Coordinatore Dottorato **Relatore**

Prof. Pietro Cortelli **Dott. Valerio Carelli**

Esame finale anno 2012

This PhD Thesis is dedicated to the memory of Professor Pasquale Montagna, who inspired and supported this project with real love for science and understanding

INDEX

INTRODUCTION

1. MELANOPSIN RETINAL GANGLION CELLS: ROLE IN CIRCADIAN PHOTOENTRAINMENT

1.1. Discovery, anatomy and physiology

Melanopsin-containing retinal ganglion cells (mRGCs) represent a new class of photoreceptors, mainly involved in the non-image forming functions of the eye, with a crucial role in photoentrainment of circadian rhythms (Hannibal et al., 2002a; Berson et al., 2002; Hattar et al., 2002). The discovery of mRGCs came from the observation that in mice models of retinal degeneration (rd/rd and rd/rd cl) light was still producing photoentrainment of their circadian rhythms (Foster et al., 1991; Freedman et al., 1999; Lucas et al., 1999). Thus, the existence of a circadian photoreceptor was postulated and in 2002, after the identification of the melanopsin photopigment (Provencio et al., 1998; Provencio et al., 2000), converging evidences proved that a small subset of retinal ganglion cells (RGCs), intrinsically photosensitive because expressing melanopsin, projected to the hypothalamic suprachiasmatic nucleus (SCN) through the retino-hypothalamic tract (RHT) (Gooley et al., 2001; Hannibal et al., 2002a,b; Hattar et al., 2002; Berson et al., 2002). The RHT was a well-known anatomical pathway connecting the eye to the SCN (Moore and Lenn 1972; Sadun et al., 1984; Moore et al., 1995), the master biological clock, but with the discovery of mRGCs its origin from a subset of RGCs was finally proven. Circadian rhythms are biological rhythms characterized by an intrinsic period of about 24 hours and the SCN represent the master circadian clock deputed to the generation of an endogenous circadian rhythm and synchronization of all the peripheral oscillators. The process by which circadian rhythms are synchronized to the light-dark cycle is named photoentrainment (Peirson et al., 2005). Many biological functions present circadian oscillations during the 24-hours, mainly hormons but also sleep-wake cycle and cognition.

Melanopsin-expressing RGCs represent about 1% of the RGCs in humans (Hannibal et al., 2004a). They are preferentially located in the parafoveal and far nasal regions of the retina (Hannibal et al., 2004a; Dacey et al., 2005). These cells are characterized by a large soma (15-20μm), with a central nucleus, and, in humans, are located in about equal proportions in the retinal ganglion cell (**Figure 1A**) and inner nuclear layers (**Figure 1B**).

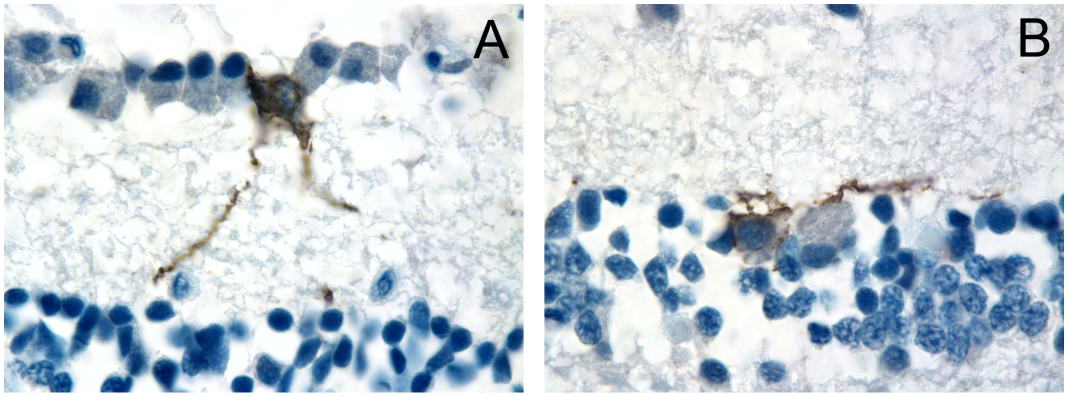


Figure 1. Postmortem human retina, immunoperoxidase staining for melanopsin with diaminobenzadine, light microscopy; Retina from a control demonstrating a melanopsin-positive retinal ganglion cell (mRGC) in the RGC layer (RGL) (A) and in the inner nuclear layer (INL) (B). In A and B dendrites extend to the "off" layer of the inner plexiform layer (formalin-fixed, paraffin embedded 5 µm section, counterstained with Mayer's hematoxylin, magnification 1000x). In both cells a centrally located nucleus and peripherally-stained soma are visible (La Morgia et al., 2012a).

The mRGCs are currently classified in subtypes, based on the levels of melanopsin expression, dendritic field harborization, physiology and central projections (Baver et al., 2008; Ecker et al., 2010, Schmidt et al., 2011a,b). The three most characterized subtypes are the M1 mRGCs, which stratify in the outermost sublamina of the inner plexiform layer (IPL), the M2 stratifying in the innermost sublamina of the IPL and the bistratified M3 with dendrites in both inner and outer sublaminae. The M4 cells have the largest soma and dendritic fields, whereas the M5 type have small and highly branched arbors and cannot be stained with melanopsin antibody, but retain a weak intrinsic response to light (**Figure 2**).

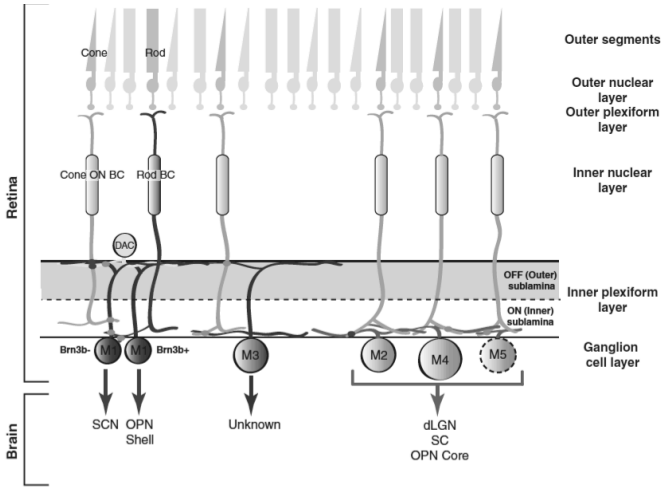
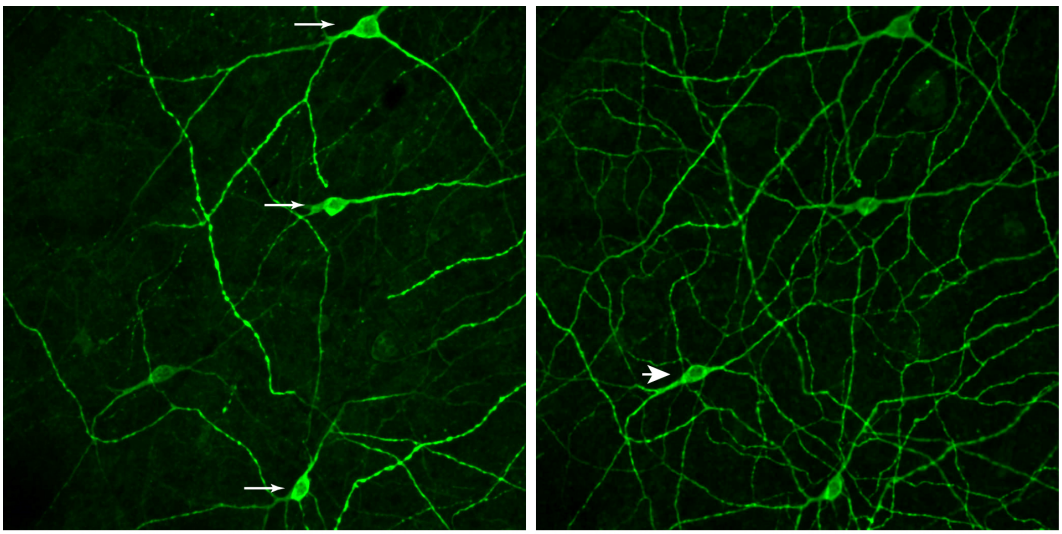


Figure 2. Schematic representation of different subtypes of mRGCs and their projections to the CNS (modified from Schmidt et al., 2011b)

The pigment is expressed primarily in the membrane of the soma and dendrites and even in the axons running within the retinal nerve fiber layer (**Figure 1**). The large and embricated dendritic fields constitute a photoreceptive net in the retina (Provencio et al., 2002) (**Figure 3**).

The mRGCs are characterized by unique photoreceptive properties. In fact, they are intrinsically photosensitive, i.e. depolarize in response to light stimuli even if they are completely isolated from the surrounding retinal structures, and use a rhabdomeric photoreceptive system, similar to invertebrates (Isoldi et al., 2005; Do and Yau, 2010). They are maximally sensitive to short wavelength blue light (peak response at 480 nm) having a sustained response to light, which persists also when the light is switched off (Foster, 2005; Do and Yau, 2010). Although they are independently functioning photoreceptors, they also receive inputs from rods and cones, through amacrine and bipolar cells (Viney et al., 2007). This organization allows the input from several types of retinal cells leading signals from the classical photoreceptors, rods and cones, constituting an "irradiance-detector" system in the eye (Viney et al., 2007; Jusuf et al., 2007; Schmidt and Kofuji, 2009, 2010; Lall et al., 2010).

The phylogenic and ontogenic importance of this cellular system is demonstrated by the fact that the melanopsin photopigment was discovered in many invertebrate organisms and that in rodents these cells start to respond to light at post-natal day 0, before the classic photoreceptors, i.e. rods and cones, start to function (Hannibal and Fahrenkrug, 2004b; Sekaran et al., 2005; Tu et al., 2005; Koyanagi and Terakita, 2008; Davies et al., 2010; González-Menéndez et al., 2010). The importance of this cellular system is also supported by the observation that in the subterranean blind mole rat the circadian photoentrainment persists, thanks to the residual presence of mRGCs projecting to brain structures deputed to control circadian rhythms, notwithstanding the involution of the conventional visual pathway (Cooper et al., 1993).



Focus on inner melanopsin RGCs and their processes located in the GCL in human retina (arrow)

Focus on outer melanopsin RGCs and their processes located in the INL (arrowhead)

Figure 3. Photoreceptive net in human retina (La Morgia et al., 2012a)

1.2. Projections and biological functions: relevance for circadian photoentrainment

The central projections of mRGCs are extensive, suggesting their influence on many non-image forming functions of the eye (Hattar et al., 2006; Vandewalle et al., 2009a; Benarroch, 2011). The primary projection of mRGCs is the SCN, through the RHT, instrumental in mediating the photoentrainment of circadian rhythms. The RHT uses glutamate as well as aspartate, PACAP and substance P as neurotransmitters (Golombek and Rosenstein, 2010; **Figure 4**). The SCN is the main target of mRGCs and may be subdivided in two main areas: 1) the core or ventrolateral area, which contains neurons releasing vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP) and receives most afferents from the retina 2) the shell or dorsomedial area, which contains neurons releasing arginine vasopressin (AVP) and calretinin and receives projections from the ventrolateral and limbic areas (Golombek and Rosenstein, 2010; Hannibal et al., 2006a; **Figure 5**). The neurons in the core region express clock genes such as Per1 and Per2 whose expression is regulated and activated by light. The communication between the core and shell of the SCN allows an oscillation with a coherent period of the SCN.

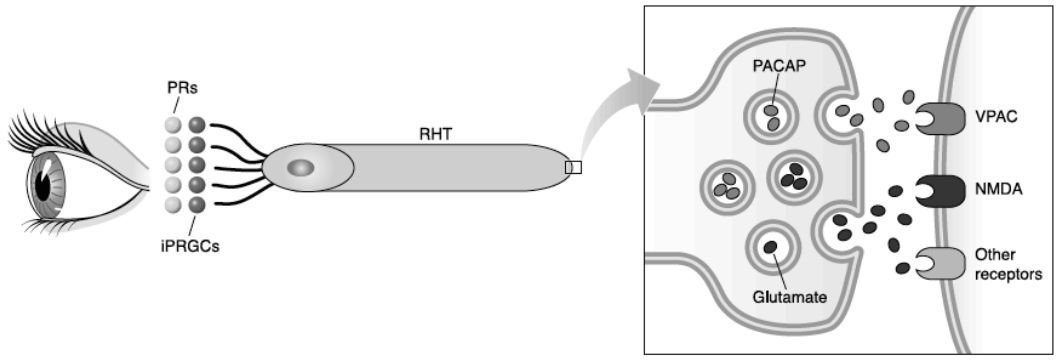


Figure 4. Representation of the RHT originating from the retina and innervating the SCN using Glutamate and PACAP as neurotransmitters (modified from Golombek and Rosenstein, 2010)

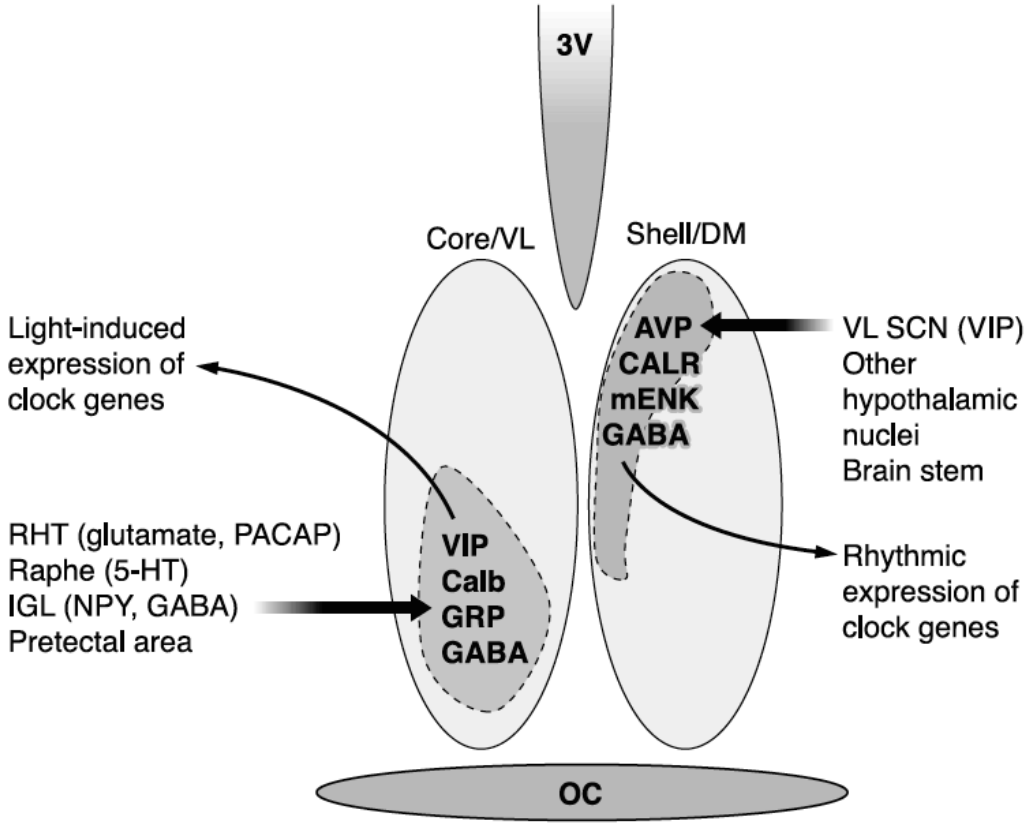


Figure 5. Anatomical and biochemical organization of the SCN (modified from Golombek and Rosenstein, 2010)

Moreover, a multisynaptic pathway originating from the retina innervates the clock from the ventral lateral geniculate nucleus and the thalamic intergeniculate leaflet using Neuropeptide Y and GABA as neurotransmitters (Golombek and Rosenstein, 2010). A further important projection of the mRGCs is to the olivary pretectal nucleus, by which they constitute the afferent arm of the pupillary light reflex, contributing mainly its sustained component (Kawasaki and Kardon, 2007). The widespread projections of mRGCs include many other key structures involved in

sleep regulation, and in particular the hypothalamic ventrolateral preoptic nucleus (VLPO), which contains sleep-active neurons, the lateral hypothalamic area, and subcortical areas related to arousal (brainstem and thalami) (Vandewalle et al., 2007; Vandewalle et al., 2009a). In fact, recent functional Magnetic Resonance Imaging (fMRI) studies suggested an important role of light in modulating cognitive functions and emotions (Vandewalle et al., 2009a). In particular, blue light increases activity, within the first few seconds of exposure, in left hippocampus, thalamus and amygdala during a working memory task (Vandewalle et al., 2007). Moreover, these cells regulate, through a polysynaptic pathway, the melatonin synthesis by the pineal gland and its suppression induced by light exposure (Brainard et al., 1983; Thapan et al., 2001; Brainard et al., 2001) (**Figure 6**). Moreover, PET studies demonstrated that light influences cortical regions involved in attention such as the occipito-parietal attention network (Perrin et al., 2004).

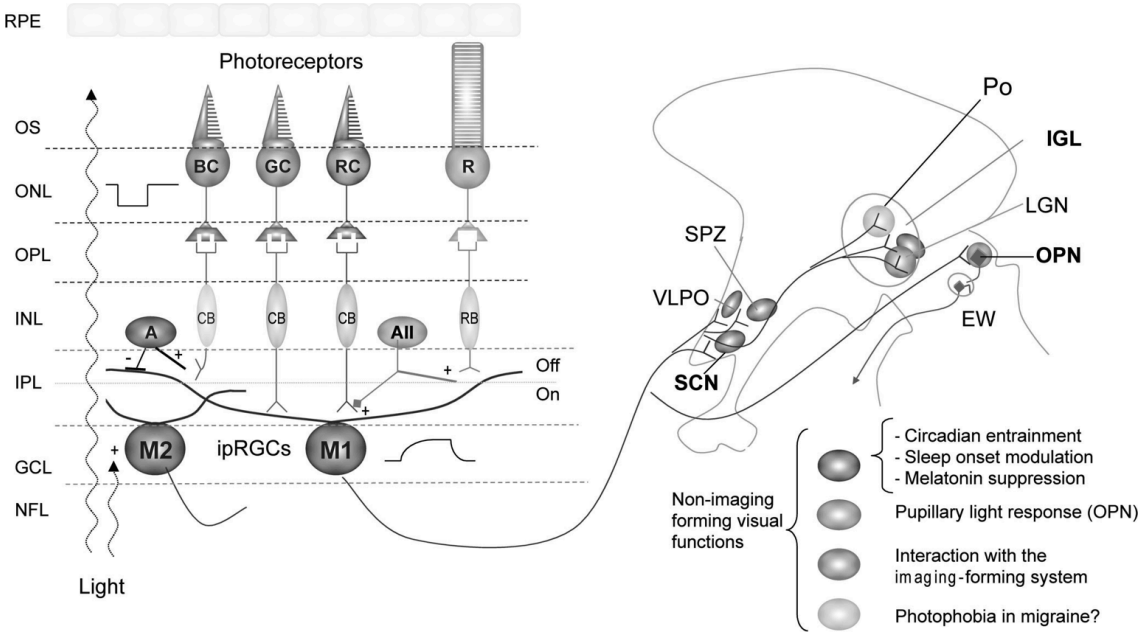


Figure 6. Main projection of mRGCs and biological functions of mRGCs are shown with particular reference to non-image forming visual functions (modified from Benarroch, 2011)

The mRGCs also target the lateral habenula along the limbic circuitry, and the intergeniculate leaflet of the thalamus involved in non-photic regulation of circadian rhythms (Hannibal et al., 2006a, 2006b; Hattar et al., 2006). Overall, these cells contribute to many non-image forming functions of the eye and have an important role in modulating circadian rhythms, alertness, sleep, cognition and emotion (Vandewalle et al., 2007, 2009a, 2010; Schmidt et al., 2009).

Even if most mRGC projections are to brain nuclei involved in non-image forming visual functions recent studies breake this paradigm and show that mRGCs may also project to structures involved in visual perception (Dacey et al., 2005; Ecker et al., 2010; Brown et al., 2010). By using a Cre-recombinase based melanopsin reporter in a mouse model, it was shown that mRGCs innervate the superior colliculus and dorsal lateral geniculate nucleus, both nuclei being retinotopically organized for object localization and discrimination (Ecker et al., 2010; Brown et al., 2010). In a further experiment, by using mice lacking classic rod/cone photoreception, it has been also shown that these animals maintain measurable visual acuity (Ecker et al., 2010; Brown et al., 2010). Moreover, mRGCs, through the projections to dura-sensitive neurons in the posterior thalamus, play an important role in the exacerbation of migraine headache by light (Noseda et al., 2010, Noseda and Burstein, 2011).

Interestingly, recent data demonstrated that distinct subtypes of mRGCs have different target in the SCN. In fact, the M1 subclass, with the highest melanopsin level expression and the highest light sensitivity is predominantly responsible for circadian photoentrainment and PLR regulation. In particular the Brn3b-positive type 1 mRGCs project to all M1 targets except the SCN, thus mainly contributing to the PLR through their projections to the OPN. At difference, the Brn3b-negative type 1 mRGCs are the main contributors for circadian photoentrainment. The non-M1 subclasses, characterized by lower melanopsin expression and less light sensitivity, may be more relevant for the image-forming and other functions like emotion modulation through projections to amygdala, limbic circuitry and periacqueductal gray (Schmidt et al., 2011a,b).

The widespread projections of mRGCs to multiple structures of the brain underlie the important role they play in physiology. The extensive influence of this parallel visual system on many biological functions provides a unique opportunity to study the impact of light and the role of the eye in human pathology.

2. MELANOPSIN RETINAL GANGLION CELLS AND SLEEP

2.1. Circadian and homeostatic regulation of sleep

Sleep is regulated by the interaction of homeostatic and circadian processes. The homeostatic process (S) can be defined as the increase of propensity to sleep as a function of the duration of prior waking and its dissipation during sleep (Borbély, 1982; Borbély and Tobler, 2011). The principal neurophysiological marker of process S is the Slow Wave Activity (SWA) during NREM sleep. The circadian process (C) controls the distribution of wake and sleep throughout the 24 hours (Daan et al., 1984; Acherman and Borbely, 2003) and depends on the activity of the suprachiasmatic nucleus (SCN), the master circadian pacemaker. In fact, it has been documented that damage of the SCN deranges the circadian regulation of sleep (Saper, 2005a,b). The intrinsic period of the SCN is not exactly 24 hours and requires daily adjustment to the light-dark cycle, a process known as photoentrainment. The light represents the main "time giver" (zeitgeber) for the synchronization of circadian rhythms and light information reaches the SCN from the mRGCs through the RHT (Berson, 2003).

The homeostatic and circadian processes operate in parallel, but interact and influence each other (Cajochen, 2010). In fact, many cerebral structures with a crucial role in homeostatic sleep regulation, such as the VLPO, are modulated by circadian influences and by direct projections from the mRGCs (Dijk and Archer, 2009). Moreover, the SCN interacts with the ventral and the dorsal subparaventricular zones, and with the VLPO through the dorsomedial nucleus of the hypothalamus (DMH) (Saper, 2005a,b). This nucleus is important for integrating circadian rhythms and sleep, motor activity, feeding and corticosteroid secretion (Saper, 2005a, 2005b). The interaction between the circadian and homeostatic processes is also reinforced by the observation of a different homeostatic regulation in morningness/eveningness types, which are characterized by a different timing of sleep, i.e. the chronotype (Mongrain et al., 2006, 2007). Moreover, emerging data support a non-circadian role of the so-called clock genes, the molecular machinery of the SCN, whose cyclic expression controls the circadian rhythm of many biological functions (Rosenwasser, 2010). In fact, homeostatic deregulation may be secondary to circadian clock disruption, either involving clock genes, or SCN or melanopsin itself (Franken and Dijk 2009; Dijk and Archer, 2009, 2010). Abnormal homeostatic phenotypes have been documented in mice lacking the core clock genes, *Cry1* and

Cry2 and in mice homozygous for the *Bmal1* deletion and *Clock* mutation (Franken and Dijk, 2009). Similarly, the variable number of tandem repeats polymorphism in the *Per3* gene, linked to extreme diurnal preference (eveningness) and delayed sleep phase syndrome (Archer et al., 2003), influences EEG markers of the homeostatic process, such as SWA (Viola et al., 2007; Vandewalle et al., 2009b). Furthermore, lesions of the SCN may produce abnormalities in homeostatic aspects of sleep regulation (Mistlberger, 2005). Conversely, the homeostatic status may influence the circadian variation of different cognitive functions and sleep variables in humans, as well as the expression of clock genes may present sleep-wake cycle dependent changes (Franken and Djik 2009). Overall, the impact of clock genes on metabolism, mood, cognition and sleep is extremely wide and may explain the important contribution of the circadian process in regulating different aspects of sleep. Finally, the sleep features of the subterranean mole rat, Spalax ehrenbergi, are very similar to other non-subterranean mammals, suggesting that the maintenance of the RHT system may be sufficient to drive a normal photoentrainment of the sleep-wake cycle, even in the absence of eyes as organ for vision (Tobler and Deboer, 2001).

2.2. Role of mRGCs in sleep

Recently, much emphasis has been given to the role that mRGCs may have in the specific modulation of sleep. In fact, Lupi and coauthors demonstrated that the genetic ablation of the melanopsin gene (*Opn4*) in mice abolishes the light-mediated induction of sleep. This sleep induction is related to the activation of sleep-promoting neurons in the VLPO and superior colliculus, as demonstrated by the strong increase in Fos espression induced by light exposure, suggesting that melanopsin-based photoreception is necessary and sufficient for sleep regulation (Lupi et al., 2008). Sleep in these mice is induced after 7-10 minutes of exposure to light at high irradiance (more than 50 $\mu W/cm^2$) and this response seems independent to rods/cones contribution (Lupi et al., 2008). Similarly, Altimus and coauthors demonstrated that mRGCs play a direct role in light-induced modification of EEG activity in mice (Altimus et al., 2008). These authors, at difference with Lupi and colleagues, showed that both rod-cone and melanopsin-based photoreception are necessary for exerting the full effect of light on sleep regulation (Altimus et al., 2008). Further studies support a relevant role of mRGCs in the regulation of sleep homeostasis (Tsai et al., 2009). Indeed, the SWA, a marker of homeostatic drive, was significantly reduced after sleep deprivation in the Opn4 $^{-/-}$ mice (Tsai et al., 2009). In this study the authors also showed that time of day modulated the acute effects of light and dark only in the Opn4$^{-/-}$ mice, suggesting that melanopsin compensates for circadian changes in rod-cone sensitivity. Moreover, melanopsin influences EEG

activity modifications induced by dark pulses, having an alerting effect (Tsai et al., 2009). The brain areas implicated in the acute effects of light on sleep-promoting neurons are the SCN and VLPO, as demonstrated by the 50% reduction in the activation of SCN neuronal activity after light pulse in the $Opn4^{-/-}$ mice and the light-induced c-fos activation in the VLPO area (Tsai et al., 2009). The preminent role of mRGCs in regulating alertness/sleep is also supported by recent brain functional magnetic resonance studies showing that brief light exposure to blue light induces a significant activation of brainstem structures related to arousal regulation and this effect may depend also on circadian phenotype (Vandewalle et al., 2007, 2011a).

Overall, these findings point to an important role of melanopsin in modulating, independently, not only the circadian aspect of sleep but also its homeostatic component. This hypothesis is well supported by the observation that different circadian phenotypes are characterized by distinguishable homeostatic regulation (Mongrain et al., 2006, 2007). Given that melanopsin exerts a fundamental role in photoentrainment of circadian rhythms and that chronotypes ('morningness'/'eveningness') reflect the ability to be entrained to the light-dark cycle, it is reasonable that genetic polymorphic variants in the melanopsin gene may play a relevant role in modulating the circadian phenotype. At this regard, the recently described association of a missense variant (P10L) of the *OPN4* gene with the seasonal affective disorder (Roecklein et al., 2009) may well support this hypothesis. Seasonal affective disorder (SAD) is characterized by recurrent depression during the short days and long nights of fall and winter. An abnormal responsiveness to light has been suggested to be part of the mechanism leading to the seasonality of the disorder, which results in a possible circadian misalignment, and explains the efficacy of light therapy, in particular the blue light in this disorder (Glickman et al., 2006; Lewy, 2009a; Lewy et al., 2009b).

3. MELANOPSIN RETINAL GANGLION CELLS IN HUMAN DISEASES

3.1. Melanopsin RGCs in human diseases

There are only a limited number of studies on the melanopsin system in human diseases.

As pointed out previously, the discovery of mRGCs came from the observation that in mice with extensive loss of rods and cones light was still able to photoentrain circadian rhythms (Foster et al., 1991; Freedman et al., 1999; Lucas et al., 1999).

In humans, this was reflected by the observation that patients suffering blinding disorders with either extensive damage of rods and cones or different types of optic neuropathy maintained the melatonin suppression response induced by light and the light sensitivity (Czeisler et al., 1995; Pèrez-Rico et al., 2009; Zaidi et al., 2007).

Differently, in other ocular disorders, such as glaucomatous optic neuropathy, mRGCs may be lost leading to the occurrence of circadian rhythms dysfunction and melatonin suppression response abnormalities (Drouyer et al., 2008; Pèrez-Rico et al. 2010).

Moreover, a possibile relevant role of mRGCs in mood disorders, and in particular in SAD, is suggested by the efficacy of blue light therapy and by the previously mentioned observation that a genetic variant in the *Opn4* gene was associated with SAD (Lewy et al., 2006, 2009a,b; Roecklein et al., 2009).

A further role of mRGCs in human disorders has been recently suggested in the exacerbation of migraine induced by light (Noseda et al., 2010, 2011). In this study, in fact, photosensitivity during migraine was present in blind migraineous patients with light perception, but absent in blind migraineous patients without light perception. Indeed, the authors of this study demonstrated that the activity of the dura-sensitive thalamocortical neurons, which are part of the nociceptive pathway underlying migraine pain, is modulated by light through the projections received from mRGCs (Noseda et al., 2010).

3.2. Melanopsin RGCs in hereditary optic neuropathies

At difference with retinal diseases characterized by extensive and selective loss of rods and cones, a class of inherited optic neuropathies, such as Leber's hereditary optic neuropathy (LHON) and dominant optic atrophy (DOA), is characterized by selective loss of RGCs, thus representing an ideal model for studying a pathology potentially affecting mRGCs.

Previous to this PhD project we studied the mRGCs system in LHON and DOA by melatonin suppression test by light in affected LHON and DOA patients compared to controls, and by immunohistochemistry in post-mortem retinal and optic nerve specimens from LHON patients and controls. Overall, this study revealed that mRGCs were relatively spared and still able to sufficiently support circadian photoentrainment (La Morgia et al., 2010) (see annex 1). In fact, these patients had a melatonin suppression response induced by light comparable to controls and the analysis of retinal and optic nerve specimens revealed that mRGCs were preferentially spared compared to regular RGCs (La Morgia et al., 2010; Figure 7-8). The observation that mRGCs are spared in mitochondrial optic neuropathies explains the maintenace of the PLR reflex in these patients, an odd clinical feature known for a long time but never fully explained (La Morgia et al. 2010, 2011) (see annex 1, 2).

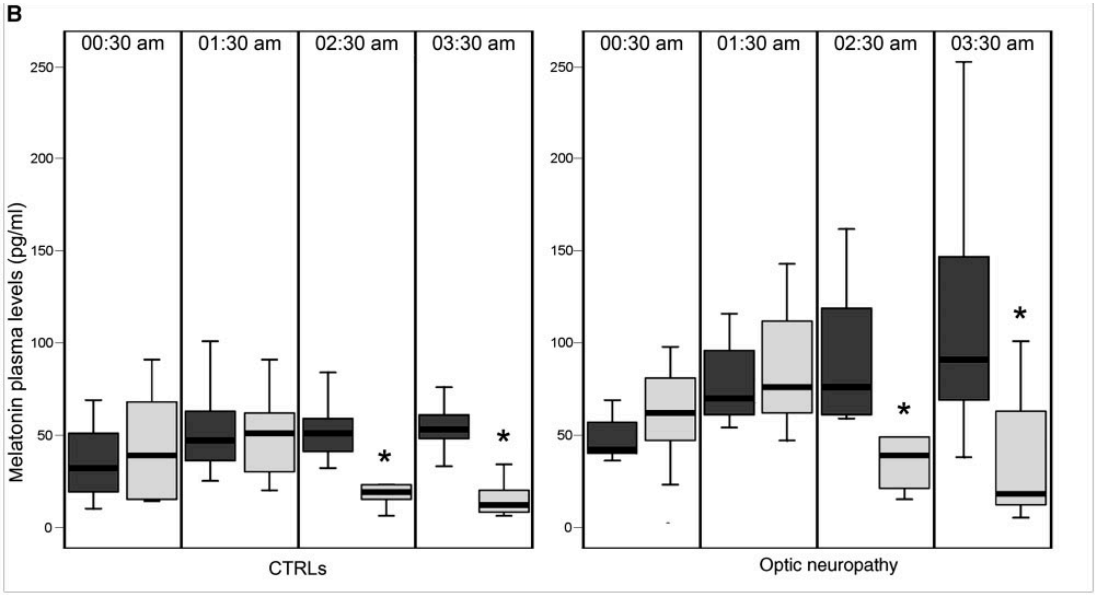


Figure 7. Melatonin suppression test results in controls and optic neuropathy patients (La Morgia et al., 2010)

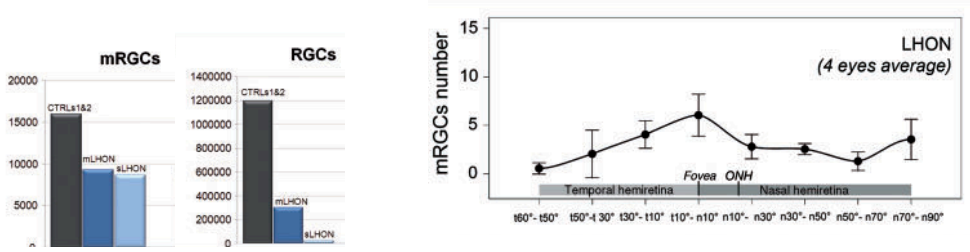


Figure 8. Left panel: immunohistochemical analysis on post-mortem retinas revealed that mRGCs are preferentially spared in comparison to the massive loss of regular RGCs in LHON patients. Right panel: distribution of mRGCs showed that they are more concentrated around the fovea (La Morgia et al., 2010)

Other authors subsequently confirmed these findings (Kawasaki et al., 2010; Moura et al., 2011; Storoni et al., 2011). Furthermore, a mice model with rotenone-induced optic neuropathy mimicking LHON was also shown to maintain the PLR (Zhang et al., 2006). Complexively, these observations suggest that mRGCs have specific metabolic properties that make them more resistant to neurodegeneration in mitochondrial optic neuropathies. This is also supported by studies showing increased resistance of mRGCs to different kind of injuries, such as for example optic nerve transection (von Bussman et al., 1993; Robinson and Madison, 2004; Li et al., 2008). Von Bussman and colleagues reported that a small subset (about 1%) of RGCs survived to optic nerve transection and these cells had large bodies, intensively stained with cytochrome oxidase (COX), pointing to mRGCs (Von Busman et al., 1993). Subsequently, Li and colleagues substantiated that mRGCs are indeed spared after optic nerve transection (Li et al., 2008). Other evidences of mRGCs robustness are provided by studies on cell toxicity to monosodium glutamate (Chambille and Serviere, 1993; Hannibal et al., 2001).

The "special" robustness of mRGCs to different stressful insults remains an open question, which needs specifically designed studies. In this PhD project the analysis of the mRGCs system in hereditary optic neuropathies has been further expanded.

Overall, optic neuropathies are a general paradigm to investigate a possible pathology affecting mRGCs, following the assumption that the specific involvement of RGCs in human diseases has the potential to affect also the melanopsin-expressing RGCs.

4. OPTIC NERVE INVOLVEMENT IN AGING AND NEURODEGENERATIVE DISORDERS.

4.1. Aging

An age-related loss of axons in the optic nerve has been documented in different histopathological studies (Dolman et al., 1980; Johnson et al., 1987; Balazsi et al., 1984). In particular, Johnson and coauthors found a similar loss of axons in the central and peripheral portions of the optic nerve without any specific regional pattern (Johnson et al., 1987). Similarly, more recent optical coherence tomography (OCT) studies demonstrated an age-related reduction of retinal nerve fiber layer (RNFL) thickness (Budenz et al., 2007; Feuer et al., 2011). The decline of the average RNFL thickness documented by these two studies was 2.0 and 2.4 μm respectively for every decade of age. This decline, according to Feuer and colleagues, is not a homogeneous phenomenon, showing a preferential loss of RNFL in the superior quadrant (Feuer et al., 2011). The temporal and inferior quadrant showed significantly less evident decline of retinal nerve fibers compared to the superior (Feuer et al., 2011). Overall, these studies document an age-related loss of axons in the optic nerve that becomes relevant in depleting the optic nerves from the age 60.

4.2. Alzheimer Disease and Mild Cognitive Impairment

Complex visual complaints, including difficulties in reading or finding objects, depth perception, perceiving structure from motion, color recognition and contrast sensitivity abnormalities are frequent in Alzheimer Disease (AD), and can affect up to 40% of AD patients (Mendez et al., 1990; Guo et al., 2010; Sadun et al., 1987; Katz and Rimmer, 1989a; Jackson and Owsley, 2003).

The presence of these abnormalities may be related to a primary retinal degeneration, as well as to a damage of the primary visual and visual associative areas. Since 1986 it was shown by Hinton and colleagues that in post-mortem retinas and optic nerves of AD patients there was loss of RGCs and related axons, involving preferentially the M-cell system, in the apparent absence of neurofibrillary or amyloid angiopathy (Hinton et al., 1986; Sadun and Bassi, 1990) (**Figure 9**).

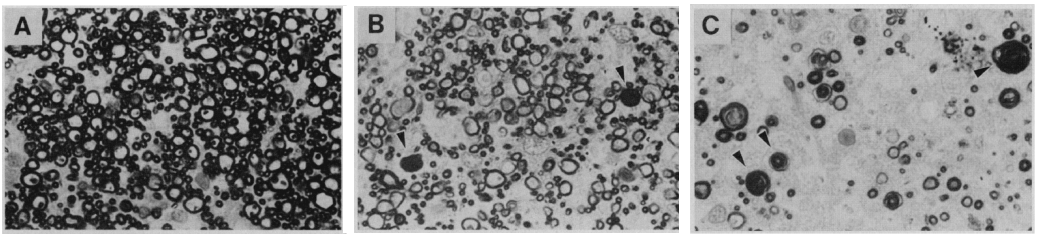


Figure 9. Optic nerves stained by p-Phenylenediamine method are shown. A. Normal control. B and C. Alzheimer optic nerves showing moderate (B) and severe (C) degenerated profile with looser axonal packing in the severe case (Hinton et al., 1986)

After this report, other authors showed the presence of optic nerve degeneration, being more evident in the superior and inferior quadrants and in the foveal region in post-mortem tissues of AD patients (Blanks et al., 1989; 1996a; 1996b). Age-related amyloid deposition in RGCs and RNFL has been documented in the human retina from normal individuals (Löffler et al., 1995), as well as evidence of retinal Aβ deposition has been reported in transgenic mouse models of AD (Ning et al., 2008; Perez et al., 2009; Dutescu et al., 2009; Liu et al., 2009). Further, the presence of amyloid plaques has been detected in whole-mounted post-mortem retinas of AD patients stained with curcumin and anti-Aβ42 antibodies (Koronyo-Hamaoui et al., 2011) (**Figure 10**).

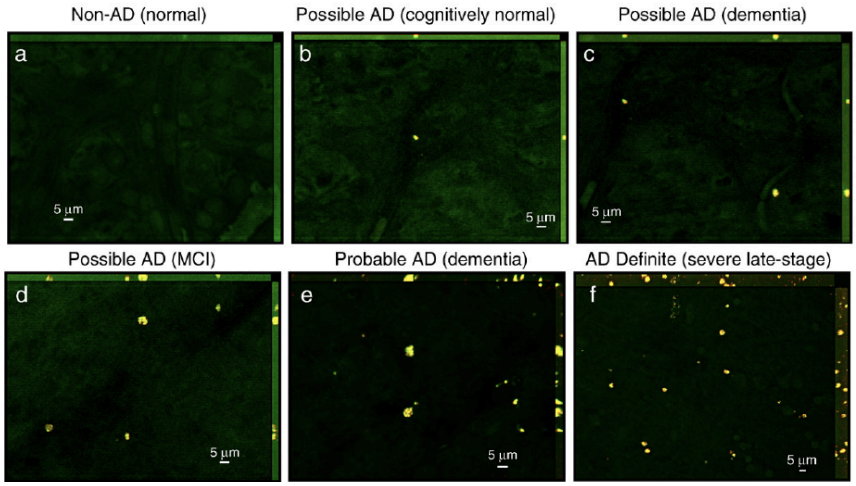


Figure 10. a-f Whole-mounted retinas from normal individuals and possible AD patients stained with curcumin anti-Aβ42 antibodies. Aβ plaques cannot be detected in cognitively normal individuals (a); in patients with severe-stage AD abundant Aβ plaques are visible (f) (modified from Koronyo-Hamaoui et al., 2011)

More recently, the use of OCT allowed objective measurements of RNFL and macular thickness for in-vivo quantitative evaluation of RGCs loss in AD. Parisi and coauthors in 2001 found significant and generalized RNFL thinning and pattern electroretinogram (pERG) abnormalities in 17 AD patients (Parisi et al., 2001, 2003).

Similarly, in 2006, Iseri and coauthors demonstrated a significant thinning of average RNFL, as well as thinning of the superior, inferior and nasal quadrants, and reduction of macular volume in 14 AD patients compared to controls, in the absence of visual evoked potentials (VEPs) abnormalities. The reduction of macular volume was correlated to the degree of cognitive impairment (Iseri et al., 2006). Significant thinning of the RNFL in the superior quadrant and decreased blood vein flow in the retina were also reported in early AD cases (Berisha et al., 2007). Moreover, also patients with amnestic mild cognitive impairment may present a diffuse reduction of the RNFL, as assessed by OCT, suggesting that optic nerve involvement may be a pre-clinical sign of neurodegeneration in AD (Paquet et al., 2007; Kesler et al., 2011). More recently Lu and coauthors demonstrated RNFL thinning of the superior and inferior quadrants in 22 AD patients (Lu et al., 2010). The preferential involvement of the superior and inferior quadrants is compatible with the seminal observations of a preferential involvement of the magnocellular system in AD (Hinton et al., 1986; Sadun and Bassi, 1990), as well as with the electrophysiological findings. This pattern resembles the optic neuropathy occurring in glaucoma (Brusini and Johnson, 2007), which interestingly has a higher incidence in AD patients (Bayer et al., 2002a,b). However, the link between AD and glaucoma remains elusive and pathogenic mechanisms linking the two conditions are controversial (Wostyn et al., 2009, 2010).

Further, Kesler and coauthors demonstrated a significant decrease of RNFL thickness in both MCI (n=24) and AD (n=30) patients compared to controls, more evident in the superior quadrant for AD, and in the inferior for both MCI and AD patients (Kesler et al., 2011). A summary of OCT findings in AD is reported in **Table 1**.

	OCT	N.patients	MMSE	Results
Parisi V et al. 2001 Clinical Neurophysiol	Stratus	17 AD 14 controls	11-19	↓avg RNFL ↓RNFL thickness in all 4 quadrants no correlation with MMSE
Iseri PK et al. 2006 J Neurophthalmol	Stratus	14 AD 15 controls	18.5 ± 6.3	↓avg RNFL thickness ↓sup/inf/nasal RNFL ↓macular volume
Paquet C et al. 2007 Neuroscience letters	Stratus	23 MCI, 14 mild AD 12 sev AD	mild AD (20-25); severe AD (11-19)	↓avg RNF thickness in MCI, mild and severe AD
Berisha F et al. 2007 IOVS	Stratus	9 AD 8 controls	24 (17-30)	↓sup RNFL thickness abnormal blood flow rate
Lu Y et al. 2010 Neuroscience letters	Stratus	22 AD 22 ctrls	not specified	↓avg RNFL ↓sup/inf RNFL thickness
Kesler A et al. 2011 Clin Neurol Neurosurg	Stratus	24 MCI 30 AD 24 ctrls	28.1 ± 2.1 (MCI) 23.6 ± 4.3 (AD)	↓avg RNFL in MCI and AD ↓inf RNFL in MCI and AD ↓sup RNFL in AD

Table 1. OCT findings in AD and MCI patients.

The presence of retinal degeneration in AD is also supported by electrophysiological investigations with VEPs and pERGs recordings, which demonstrated an abnormal RGCs function, even at early stages of the disease (Visser et al., 1976, 1985; Coben et al., 1983; Pollock et al., 1989; Katz et al., 1989b; Parisi et al., 2001; Trick et al., 1989; Sartucci et al., 2010; Krasodomska et al., 2010). Interestingly, Krasodomska and coauthors studied 30 early AD patients, with Mini-Mental score more than 21 and duration of the disease less than 2 years, reporting an increased implicit time of the P50-wave and amplitude reduction of the P50 and N-95 in pERG examination, an increased latency of P-100 wave as well as a significant prolonged retino-cortical time (RCT) in pattern VEPs (Krasodomska et al., 2010). Sartucci and coauthors, using both chromatic and luminance visual stimuli in 15 early AD cases, described abnormal pERG and VEPs responses with normal retinocortical conduction time, compatible with a primary dysfunction of the magnocellular pathway in AD (Sartucci et al., 2010). These findings are in accordance with the observation of a prevalent deposition of amyloid plaques and neurofibrillary tangles in the primary visual cortex prevalent in the M-pathway described in AD (Hof et al., 1997) and propose that the involvement of the retina may be secondary to a retrograde axonal degeneration (Sartucci et al., 2010).

Overall, these findings suggest an early involvement of the visual system in the neurodegenerative process in AD, prompting the screening of retinal abnormalities in mild cognitive impairment patients. It is still controversial whether the visual

problems are a consequence of a primary retinal or cortical involvement in AD, or both. In support of a primary retinal contribution to the occurrence of visual dysfunction in AD is the evidence that melatonin suppression response induced by light in AD patients is impaired, suggesting the presence of an abnormal mRGCs-RHT-SCN axis (Ohashi et al., 1999).
The presence of a specific involvement of the mRGCs system may also explain the occurrence of impaired pupillary light reflex described in AD, even if this has been also related to the presence of a cholinergic defect (Fotiou et al., 2007, 2009).

4.3. Parkinson disease

In the last few years emerging evidences support the involvement of the retina and the optic nerve in the neurodegenerative process in PD, explaining the occurrence of visual dysfunction in PD (Bodis-Wollner 2009; Archibald et al., 2009, 2011a). Visual problems, including abnormalities in colour vision, may occur early and are by some authors proposed as markers, among the nonmotor signs, with the best discriminatory power in distinguishing early PD from controls (Diederich et al., 2010). Visual dysfunction, and in particular colour vision abnormalities seem to occur more likely in PD patients with Rem Behavior Disorder (RBD) than without RBD, and they are also described in idiopathic RBD (Postuma et al., 2008, 2009), suggesting that different subgroups of PD patients may have different clinical expression of non-motor symptoms. In particular, impaired visual acuity (Jones et al., 1992; Matsui et al., 2006), abnormal contrast sensitivity (Regan and Neima, 1984; Bodis-Wollner et al., 1987; Diederich et al., 2002), colour vision abnormalities (Price et al., 1992; Silva et al., 2005) and optic nerve degeneration have been described in PD (Hunt et al., 1995; Archibald et al., 2009). Furthermore, impaired visual processing has been related to visual hallucinations in PD (Meppelink et al., 2009). The defects in contrast sensitivity described in PD have been related to the dopamine deficiency in the retina, as demonstrated by the fact that many of these alterations improve after the administration of L-Dopa (Bulens et al., 1987; Hutton et al., 1993). In fact, retinal depletion of dopamine in PD patients has been described since 1988 (Nguyen-Legros, 1988, Harnois and Di Paolo, 1990; Bodis-Wollner, 1990) and shown to play a relevant role in the contrast sensitivity, VEPs and pERG abnormalities described in these patients (Bodis-Wollner and Yahr, 1978; Tagliati et al., 1996). Retinal dopamine regulates, via D1 receptors, the light adaptation process influencing the surrounding organization of the RGCs (Bodis-Wollner, 2009). Further, visual evoked potentials and pattern ERG abnormalities described in PD may be reversed by the administration of L-DOPA (Peppe et al., 1995, 1998). Moreover, the observation of reduced dopamine content in the retina of PD patients

may turn out relevant in the occurrence of circadian rhythms dysfunction in PD. In fact dopaminergic amacrine cells interact bidirectionally and influence the activity of mRGCs (Zhang et al., 2008; Vugler et al., 2007) and dopamine itself may influence the melanopsin gene expression (Sakamoto et al., 2005; Hannibal et al., 2006c). An abnormal retinal dopaminergic function may impair the integrity of the mRGCs system, possibly contributing to the occurrence of circadian and sleep problems in PD patients.

Optical coherence tomography (OCT) and Heidelberg Retina Tomograph (HRT) measurements in PD patients documented a reduction of the retinal nerve fiber layer (RNFL) thickness in both early and more advanced stages of the disease. Inzelberg and coauthors in 2004 described a significant thinning of the RNFL thickness by OCT in the inferotemporal quadrants in 10 PD patients (Inzelberg et al., 2004). Yavas and coauthors found a significant RNFL thickness reduction by HRT in the nasal, superonasal, inferonasal and inferotemporal sectors in 44 PD patients and, interestingly, the RNFL thickness was higher for patients treated with l-DOPA than with dopamine agonists, suggesting a possible neuroprotective role of levodopa therapy (Yavas et al., 2007). Similarly, Altintas and coauthors described a generalized reduction of the RNFL by OCT, which reached the significance for the superior and nasal quadrants, and of the macular thickness and macular volume in 17 PD patients compared to controls (Altintas et al., 2008). In this series foveal thickness is correlated with UPDRS score (Altintas et al., 2008). More recently Hajee and coauthors, using a Fourier-domain OCT, found a significant thinning of the superior and inferior thickness of macular inner retinal layer in 24 early PD patients (48% of the sample were drug naïve patients) (Hajee et al., 2009). These measurements did not correlate with the disease severity (Hajee et al., 2009). Moschos and colleagues, combining the OCT and multifocal ERG techniques demonstrated a significant thinning of the RNFL thickness in the inferotemporal quadrant and reduced foveal electrical activity in 16 PD patients without visual impairment (Moschos et al., 2011). Cubo and coauthors found a reduced foveal thickness in 9 PD and 9 essential tremor patients compared to controls (Cubo et al., 2010). Differently, Archibald and colleagues did not find any differences in terms of retinal thickness between 34 PD patients and 17 controls (Archibald et al., 2011b). Similarly, Aaker and coauthors did not find significant differences between PD and controls in terms of RNFL thickness, but only for macular thickness measurements (Aaker et al., 2010).

A summary of OCT findings in PD is reported in **Table 2**.

	OCT	N.patients	Results
Inzelberg et al. 2004 Vis Res	Stratus	10 PD 10 ctrls	↓inf RNFL thickness ↓temp RNFL thickness No correlation with disease duration
Yavas et al. 2007 Eur J of Opthalmol	Heidelberg Retinal Tomograph	44 PD 21 controls	↓avg, nasal, supero and infero-nasal and infero-temporal RNF thickness (patients on L-dopa have thicker RNFL)
Altintas et al. 2008 Doc Ophthalmol	Stratus	17 PD 11 controls	↓avg, sup and nasal RNFL thickness and ↓macular thickness and volume (correlation foveal thickness/UPDRS)
Hajee et al. 2009 Arch Ophthalmol	Spectral-domain OCT (macula)	24 PD (48% de-novo) 17 controls	↓ sup and inf thickness of macular IRL No correlation with severity of disease
Moschos et al. 2010 Eur J Opthalmol	Stratus	16 PD 20 controls	↓inf and temp RNFL thickness
Cubo et al. 2010 Mov Dis	Stratus	9 PD; 8 ET 9 controls	↓foveal thickness in PD and ET vs controls (thinner in the eye controlateral ete of the most affected side)
Archibald et al. 2011 Park and relat dis	Stratus	34 PD 17 controls	no differences in RNFL thickness, macular thickness and volume

Table 2. OCT findings in PD patients.

The observation that optic nerve dysfunction may occur early in the disease course is extremely important, representing a possible reliable early biomarker for the diagnosis of PD.

Interestingly, a mouse model with a spontaneous deletion in the ubiquitin C-terminal hydrolase L1 (*UCH-L1; Park5*), whose variants are associated with sporadic AD and PD, is characterized by a disturbed sleep/wake rhythm in association with a significant reduction of mRGCs in the retina (Pfeffer et al., 2012). This is direct evidence connecting the retinal pathology to the abnormalities of circadian rhythms and sleep in a model of neurodegeneration.

Overall, the involvement of the retina, and in particular of the RGCs in the neurodegenerative process in PD may allow to hypothesize a contribution of the mRGCs system in the occurrence of sleep and circadian rhythms dysfunction in PD human patients.

5. SLEEP AND CIRCADIAN DYSFUNCTION IN AGING AND NEURODEGENERATIVE DISORDERS

5.1. Aging

Sleep-related complaints are common in aging and affect up to 70% of the elderly population (Van Someren, 2000). They include difficulties in initiating and maintaining sleep with early awakenings, and changes in the quality and timing of sleep (Dijk et al., 2000; Huang et al., 2002; Bliwise et al., 2005; Wu and Swaab, 2007a; Auger and Boeve, 2011). Sleep efficiency declines progressively with age, from approximatively 86% at age 37-54 to 79% over age 70 (Bliwise et al., 2005). Changes in the sleep architecture are represented by a reduction of the total sleep time and in particular of the time spent in slow wave sleep (SWS), but also reduced density of REM sleep and loss of increase of REM sleep through the night (Neikrug and Anconi-Israel, 2010). Changes in the timing of sleep include early awakenings and early bedtime. These modifications result from both an abnormal homeostatic regulation of sleep and/or dysfunctional regulation of circadian rhythms (Huang et al., 2002; Cajochen et al., 2006). In particular, the homeostatic changes described with aging comprise a reduction of the slow-wave and spindle frequency activity during sleep, and increased awakenings during the night (Cajochen et al., 2006). The circadian rhythm-related changes reported with aging are represented by a reduction of the amplitude of core body temperature by 20-30%, melatonin and cortisol circadian rhythms, earlier bed and wake-time and phase-advance of circadian rhythms (Mirmiran et al., 1992; Dijk et al., 2000). The latter has been interpreted not to be secondary to a change of the endogenous circadian period per se but to a weakened circadian process (Cajochen et al., 2006). The occurrence of sleep and circadian abnormalities in the elderly may be determined by: a) degenerative changes affecting the retina and the optic nerve (Johnson et al., 1987; Chakravarty et al., 2010) b) reduced transmission of light, particularly blue light, through a yellowing ocular lens c) increased incidence of macular degeneration and glaucoma (Turner and Mainster, 2008; Heiduschka et al., 2010). Moreover, both in animal models and humans there is evidence that mRGCs are lost with aging (magnitude of 40% reduction) (Semo et al., 2003; Lax et al., 2011; Lupi et al. 2012; Esquiva et al., 2010). Interestingly, Lupi and coauthors demonstrated that aging rather than retinal degeneration was more important in reducing the SCN response to retinal inputs (Lupi et al., 2012). In fact, the amplitude of light-induced Fos expression in the SCN is significantly reduced in aged mice but it is similar in wild-type and retinal

degenerated mice (Lupi et al., 2012). Further, the presence of lipofuscin deposits has been demonstrated in aging human retinas (Vugler et al., 2007). All these conditions may affect the ability of the eye to synchronize circadian rhythms to the light-dark cycle. Moreover, loss of mRGCs with aging may also affect, due to the projections of these cells to the VLPO, the stability of the flip-flop switch mechanism of sleep, hence leading to increased propensity to be awake during sleep (Saper, 2005a,b). Thus, the sleep abnormalities described in the elderly may be interpreted as a consequence of a reduced circadian signal. Moreover, at the level of the SCN, neurodegenerative changes including loss of vasopressin-expressing neurons and reduced sensitivity of the clock gene machinery to light stimuli may contribute to a reduced efficiency of the photoentrainment process (Hofman and Swaab, 1994; Hofman, 2000). Furthermore, degenerative changes of the pineal gland, abnormalities of the melatonin circadian rhythm (Skene et al., 1990; Wu and Swaab, 2005), an increase in the calcium content of the pineal gland (Humbert and Pevet, 1991), abnormal sympathetic regulation of melatonin synthesis (Wu and Swaab, 2005) and decreased expression of melatonin receptors at the SCN level (Wu et al., 2007b; Sánchez-Hidalgo et al., 2009) have all been reported with aging. Finally, elderly are less exposed to light and other non-photic zeitgbers, such as food and social enviroment (Auger and Boeve, 2011). The prominent role played by the mRGCs-RHT-SCN system in the pathogenesis of circadian and sleep problems in aging is also supported by the amelioration of sleep dysfunction with light therapy (Wu and Swaab, 2007a; Dowling et al., 2008; Riemersa-van der Leck et al., 2008). Further, the fragmentation of the rest-activity rhythm with age is relevant for the occurrence of age-related cognitive deficits (Oosterman et al., 2009) and the day-to-day stability of sleep-wake rhythm is a strong predictor of cognitive disturbances (Carvalho-Bos et al., 2007), as well as the loss of sleep is a major predictor of the occurrence of dementia (Kang et al., 2009). Moreover, sleep-dependent consolidation of memory is altered with aging (Harand et al., 2012; Pace-Schott & Spencer, 2011). The management of sleep and circadian dysfunction occurring with age may be extremely important to counteract the age-related cognitive decline.

5.2. Alzheimer Disease and Mild Cognitive Impairment

The occurrence of sleep and circadian abnormalities is frequent (25-40%) in AD patients and may present early in the course of disease (Reynolds et al., 1988; Vitiello et al., 1991; Carpenter et al., 1995; McCurry et al., 1999; Van Someren et al., 1997a,b; Moran et al., 2005; Wu and Swaab, 2007a). The severity of sleep disruption often worsens while the disease progresses over the years contributing to the severity of cognitive impairment (Moe et al., 1995). Rest-activity rhythm circadian

dysfunction in AD patients compared to age-matched controls has been studied by prolonged actigraphic monitoring and is represented by fragmented sleep-wake cycle with frequent awakenings during the night and daytime naps, higher intradaily variability and less interdaily stability of the rest-activity rhythm, lower rhythm amplitude and later acrophase (Witting et al., 1990; Satlin et al., 1995; Van Someren et al., 1996, 1997a,b; Ancoli-Israel et al., 1997; Mishima et al., 1997; Hatfield et al., 2004; Harper et al., 2005, 2008; Hu et al., 2009). These changes in the rest-activity rhythms may appear early in the disease course (Hu et al., 2009). Furthermore, an irregular sleep-wake pattern and a phase advance of the sleep-wake rhythm have been reported in AD patients (Prinz et al., 1982). Polysomnographic studies also documented an abnormal macrostructure of sleep, and in particular reduced sleep efficiency and slow wave activity and longer REM sleep latency (Vitiello and Prinz, 1989; Moe et al., 1995; Yesavage et al., 2004; Cooke et al., 2006). Moreover, AD patients frequently suffer sundowning, i.e. the occurrence of motor and behavioural agitation in the late evening/night. Sundowning may be considered as a chronobiological disorder related to an abnormal circadian rhythm of the body temperature, and in particular to a phase delay of this rhythm (Volicer et al., 2001). Furthermore, lower melatonin levels, especially in patients homozygous for the APOE ε4 allele (Liu et al., 1999), reduced amplitude of melatonin circadian rhythm (Mishima et al., 1999), irregular melatonin rhythm (Uchida et al., 1996), lower cerebrospinal fluid levels of melatonin (Liu et al., 1999) as well as a significant reduction of the melatonin receptors type 1 expression characterizing the advanced stages of AD, have all been documented also in preclinical (Braak stages I-II) AD cases (Zhou et al., 2003, Wu et al., 2007b). Other examples of circadian dysfunction in AD include a phase-delay (Satlin et al., 1995; Volicer et al., 2001; Harper et al., 2001), increased amplitude (Touitou et al., 1986) and disorganization of the body temperature circadian rhythm (Okawa et al., 1991). The presence of core body temperature disorganization may predict the occurrence of more severe sleep disturbances in these patients (Satlin et al., 1995). Abnormalities of heart rate (Reynolds et al., 1995), blood pressure (Otsuka et al., 1990) and cortisol rhythm (Hatfield et al., 2004) have also been documented in AD. Interpreting the circadian and sleep dysfunction in AD, it must be taken into account a possible abnormal phase relationship between circadian rhythms, as for elderly, explaining the discordance between the phase-delay of the temperature rhythm and the reported phase-advance of sleep rhythm (Auger and Boeve, 2011).

Sleep and circadian abnormalities have also been described in mouse models of AD characterized by extracellular Aβ deposition (Wisor et al., 2005; Gimenez-Llort et

al., 2007; Sterniczuk et al., 2010). Wisor and coauthors, studying a mouse model with age-dependent deposition of amyloid in the brain, found that sleep abnormalities are related to the cholinergic deficiency, due to the accumulation of oligomeric Aβ species in the brainstem (Wisor et al., 2005). Interestingly, Sterniczuk and coauthors recently demonstrated in a triple transgenic AD mouse model that abnormalities of circadian rhythms are very early, preceding the AD pathology. In particular, the authors showed a reduced number of vasoactive intestinal polypeptide (VIP) and vasopressin (AVP) cells in the SCN of the AD mice compared to control animals (Sterniczuk et al., 2010).

Many factors may contribute to the pathogenesis of circadian rhythms abnormalities in AD patients, which include genetic factors, as the APOE genotype (Yesavage et al., 2004), neurodegenerative changes of the retina and in particular of the mRGCs specific of AD, changes of the endogenous circadian period and neurodegenerative findings at the SCN level. The neurodegenerative changes affecting the SCN described in AD patients are more pronounced and occur earlier than in age-matched controls (Swaab et al., 1985, 1987). Moreover, the neuropathological findings characteristic of AD, such as tangles and pretangles, but not neuritic plaques, and reduced AVP gene expression have been found in the SCN of AD patients (Stopa et al., 1999; Wu and Swaab, 2007a). Another factor that may contribute to the pathogenesis of circadian dysfunction in AD, is the reduced light exposure during the day (Wu and Swaab, 2007a). Many of these changes are also relevant for the occurrence of circadian dysfunction in elderly, but in AD they are more dramatic, early and severe.

The occurrence of circadian and sleep dysfunction has been documented also in about 14-59% of MCI patients, even if these data are mainly derived from subjective measures, such as sleep questionnaires (Beaulieu-Bonneau et al., 2009). In amnestic MCI patients a lower interdaily stability of the rest-activity rhythm and reduced sleep quality are associated to inadequate memory consolidation (Westerberg et al., 2010). Moreover, recent studies suggest that the presence of rest-activity rhythm abnormalities may represent a strong predictor for the development of dementia. In particular, the presence of decreased circadian activity rhythm amplitude and robustness, and delayed rhythm increases the risk of developing MCI and dementia in a 5 years follow-up study (Tranah et al., 2011). Moreover, chronic sleep restriction significantly increases the formation of amyloid plaques in an amyloid precursor protein transgenic mouse model, thus possibly playing a pathogenic role in the development of AD (Kang et al., 2009).

A summary of studies on circadian rhythms in AD is provided in **Table 3 and 4**.

	Parameters	N.patients	Results
Campbell et al. 1988	Actigraph (Cosinor analysis)		No differences between AD and controls
Witting et al. 1990 Biol Psychiatry	Actigraph for 90-168 hrs	6 young ctrls 13 old controls 12 AD hospitalized (mod-sev)	Lower IS and increased IV No significant differences between young and old controls
Satlin et al. 1995 Neurobiol of aging	Actigraph for 72 hrs Core body T cosinor analysis and IS CR	28 AD severe hospitalized 10 controls	Lower IS and increased nocturnal activity in AD Lower amplitude and later acrophase of locomotor activity and later acrophase of T' rhythm
Van Someren et al., 1999 Chronobiol Int	Actigraph for 2 weeks Non parametric methods compared to cosinor analysis	17 AD hospitalized	Reduced IS in AD IS improved by light therapy (parameter with high sensibility)
Volicer et al. 2001 Am J Psychiatr	Actigraph (cosinor and non-parametric) Core-body T (72hrs) CR only for AD	25 AD (severe) 9 controls	Reduced IS and later acrophase of rest-activity rhythm Higher temperature mesor, greater amplitude and later acrophase Later acrophase of T and locomot activity in sundowning pts
Hatfield et al., 2004 Brain	Actigraph for 28 days repeated after1 yr Non-parametric methods Cortisol rhythm Free-running	19 ctrls 27 AD not instituzionalized (mild, AD; n=13- and moderate AD; n=14)	Reduced IS and RA; higher IV in moderate AD vs controls Positive correlation MMSE-IS Heterogeneous findings Normal cortisol rhythm Substantial stability after 1 yr
Harper et al. 2005 Am J Ger Psych	Actigraph Temperature Non-parametric and cosinor analysis Costant routine	Normal young (7) Old controls (7) and severe AD (7) Pre-constant routine for 72 hrs and constant routine for 36-hrs	Higher mesor, lower amplitude and delayed phase of T in AD Reduced IS and RA and higher IV Reduced mesor and absolute amplitude
Harper et al. 2008 Brain	Actigraph for 72 hrs Temperature for 72 hrs SCN (Dorsomedial-vasopressin and neurotensin neurons) neuropathology Non-parametric and cosinor analysis	19 AD pts 8 controls	Significant loss of vasopressin neurons in AD Significant correlation between temperature rhythm and SCN degeneration Vasopressin loss → increased IV Neurotensin loss → reduced amplitude of temperature and RA of locomotor rhythm
Hu K et al. PNAS 2009	Scale-invariant activity fluctuations (driven by the SCN) Actigraph for 1 week	13 young ctrls 13 elderly ctrls 12 very old controls 13old- early stage AD 14very-old late stage AD	Reduced scale invariance of activity fluctuation in AD

Table 3. Summary of studies on circadian rhythms findings in AD patients.

	Parameters	Animal models	Results
Wisor et al. Neuroscience 2005	Actigraphic monitoring in LD and DD cycles EEG	Tg2576 mouse model (amyloidogenic swedish mutation) (age-dependent amyloid deposition)	Longer circadian period in constant darkness (secondary to cholinergic deficiency) Abnormal timing of sleep relative to the LD 12:12 cycle Lower delta power after sleep deprivation EEG power shifted to higher frequencies in NREM
Sterniczuk et al., 2010 Brain Res	Actigraphic monitoring in LD and DD cycles Free-running Period Photic phase shifting SCN Histology	Triple transgenic AD mice (Abeta and tau pathology)	Greater locomotor activity during the day and shorter free-running period before the appearance of AD pathology (enhanced aging findings) Loss of VIP and VP neurons in the SCN Normal photic phase shifting

Table 4. Summary of studies on circadian rhythms findings in AD mouse models.

5.3. Parkinson disease

The presence of sleep disturbances in Parkinson disease (PD) is an early and frequent (up to 88% of PD patients) complaint, often preceding many years the onset of motor symptoms (Thorpy and Adler, 2005). In particular, Rem-sleep Behaviour Disorder (RBD), occurring in 15-50% of PD patients, may start up to half a century before the appearance of motor signs (Claassen et al., 2010, Menza et al., 2010). The early appearance of RBD in the clinical course of PD is in accordance with the temporal and topographical sequence of ascending pathology described by Braak and coauthors (Braak et al., 2002). Moreover, the majority of PD patients have disturbances in initiating and maintaining sleep, poor quality of sleep and frequent nighttime awakenings. The increased fragmentation of night sleep is related to many factors including rigidity, difficulty in changing body position and muscle cramps, as well as medication and concomitant sleep disorders. Polysomnographic recordings of PD patients documented an abnormal sleep macrostructure with reduced sleep efficiency, total sleep time, slow wave and REM sleep and increased wake-time during the night (Chokroverty, 1996; Diederich et al., 2005; Adler and Thorpy 2005; Simuni and Sethi, 2008). Actigraphic monitoring of PD patients demonstrated an elevated nocturnal activity level and an increased movement time during the night compared to controls (van Hilten et al., 1994; Stavitsky et al., 2010). Degenerative changes affecting the brainstem structures involved in the control of sleep and wake, such as the pedunculopontine nucleus (PPN), the locus coeruleus, the raphe nucleus, the ventrolateral tegmental area, and the suprachiasmatic nucleus, may all contribute to the occurrence of sleep disturbances in these patients (Simuni and Sethi, 2008).

Furthermore, in PD patients there is evidence of an increased incidence of Restless Legs Syndrome, Periodic Limb Movements and Obstructive Sleep Apnea Syndrome compared to the general population and these sleep disorders may contribute and aggravate the sleep fragmentation and excessive daytime sleepiness during the day (Poewe and Högl 2000; Simuni and Sethi, 2008). Excessive daytime sleepiness affects up to 50% of PD patients and may be explained by the contribution of many factors, including the motor disability, the frequent concomitant occurrence of primary sleep disorders, the use of dopaminergic drugs, and also degenerative changes involving the brainstem cholinergic, serotoninergic, and noradrenergic regions, such as the PPN, locus coeruleus-subcoeruleus complex, and the reticular activating system (Boeve et al., 2007), as well as the loss of orexinergic neurons in lateral hypothalamus (Langston and Forno, 1978; Fronczek et al., 2007). This latter finding may explain the presence of narcolepsy-like features in some PD patients (Arnulf, 2005; Haq et al., 2010). However, other studies did not support the involvement of the hypocretinergic neurons in the PD neurodegenerative process in PD (Compta et al., 2009).

Circadian alterations in Parkinson disease include phase advance and reduced amplitude of melatonin circadian rhythm (Bordet et al., 2003), reduced body temperature rhythm amplitude (Cagnacci et al., 1990) and abnormalities of heart rate variability, blood pressure and rest-activity circadian rhythm (Senard et al., 1992; Pursiainen et al., 2002; Whitehead et al., 2008; Niwa et al., 2011). In particular, considering the rest-activity rhythm, PD patients present increased intradaily variability, lower peak and reduced amplitude of activity levels compared to controls (Whitehead et al., 2008). These differences are more pronounced in PD patients with hallucinations suggesting that the presence of circadian abnormalities may be limited to a subgroup of PD patients (Whitehead et al., 2008). Another study with actigraphic monitoring documented the presence of reduced rhythm amplitude in PD compared to controls (Niwa et al., 2011). However, in the evaluation of these findings many confounding factors must be taken into account, including the presence of motor artifacts and the influence of dopaminergic treatment. Interestingly, the presence of reduced amplitude and phase advance of circadian rhythm of temperature, heart rate and locomotor activity has been demonstrated also in a rat model of PD, produced by bilateral striatal 6-OHDA lesions (Ben and Bruguerolle, 2000). In this model the infusion of L-Dopa may restore, at least partially, the circadian rhythm abnormalities (Boulamery et al., 2010). More recently, circadian abnormalities of rest-activity rhythm have been documented in a *Park5* mouse model, which presented also loss of orexin neurons and mRGCs

(Pfeffer et al., 2012). Similarly, MPTP-treated monkeys presented early (before motor symptoms), persistant and relevant fragmentation and reduced amplitude of rest-activity rhythm (Vezoli et al., 2011) and transgenic ASO mice (α synuclein overexpression) showed disrupted circadian rhythm aggravated by aging (Kudo et al., 2011). Moreover, there are both clinical (Bruguerolle & Simon, 2002; Cai et al., 2010) and experimental evidences that dopamine depletion per se may affect circadian rhythms and expression of clock genes in different brain regions (Ben & Bruguerolle, 2000; Boulamery et al., 2010; Gravotta et al., 2011) and rats injected bilaterally with 6-hydroxydopamine showed a significant disruption of circadian rhythms in continuous darkness. These abnormalities are partially restored when the light-dark cycle was re-established (Gravotta et al., 2011). Overall, these findings point to a direct role of dopamine in regulating circadian rhythms.

Furthermore, in PD there is also evidence of circadian fluctuation of motor symptoms during the day, which results from the combined influence of the dopaminergic pharmacologic effects, spontaneous circadian rhythm of motor activity and circadian changes of the dopaminergic function in the central nervous system (Bruguerolle and Simon, 2002). Recently, PD has been interpreted as a neuroendocrine disorder of circadian function (Willis, 2008a). This hypothesis is based on the consideration that the nigrostriatal and circadian system are integrated and proposes that the motor impairment in PD may result from the imbalance between dopamine and melatonin neurochemical functions. Within this frame, it is suggested that the nigrostriatal and the visual system play a major role in the pathogenesis of motor symptoms in PD (Willis, 2008a), as suggested by the increased severity of motor symptoms after ocular enucleation (Willis et al., 2008b). On the contrary, both light-therapy in humans (reducing melatonin levels) and intraocular injection of dopamine in animal models of PD may improve motor symptoms in PD patients (Paus et al., 2007; Willis, 2008c).

A summary of studies on circadian rhythms in PD is provided in **Table 5 and 6**.

	Parameters	N.patients	Results
Pierangeli et al. 2001	Rectal T° SW cycle CR	7 iPD 14 MSA 8 CTRLS	Normal 24-hrs Rectal T° rhythm in PD
Bordet et al. 2003 Clin Neuropharmacol	Melatonin Cortisol Rectal T° Rest-activity (Cosinor analysis) CR	PD untreated (n=8) PD treated without LDMRC (n=10) PD treated with LDMRC (n=8) No control group	Decreased amplitude of T° in treated pts Deacreased amplitude and phase advance of melatonin rhythm in treated pts Increased daytime secretion in group III No differences in rest-activity rhythms between groups
Whitehaed et al. 2008 Mov Dis	Rest-activity (Non-parametric analysis) IS-IV-RA	50 PD (27 with allucinations and 23 without) 29 controls	Increased IV in PD ($p<0.001$) Lower M10 ($p<0.001$) Lower amplitude ($p<0.001$) Allucinators PD lower IS, greater night-time activity (L5) and reduced RA compared to non-allucinators (ANCOVA)
Niwa et al. 2011 Autonomic Neuroscience	Rhythm pattern analysis (AW2 program)*	27 PD 30 controls	Higher sleep episodes out-of-bed and wake episode in-bed in PD Reduced rhythm amplitude in PD

Table 5. Summary of studies on circadian rhythms findings in PD patients.

	Parameters	Animal models	Results
Ben V and Bruguerolle, Life Sciences 2000; Boulamery et al., Chronobiol Int 2010	Temperature Heart rate Locomotor activity (Fourier and cosinor analysis)	6-OHDA striatal injection Rats	Significant decrease of the mesor and phase advance of HR, T° and locomotor activity rhythms Significant reduction of HR rhythm amplitude Partial restoration with L-Dopa
Pfeffer et al. Neurobiology of aging 2010	Rest-activity rhythm Orexin neurons Melanopsin cells	PARK5 mouse model (UCH-L1)	Abnormal sleep/wake rhythm Loss of orexin neurons Loss of melanopsin cells
Gravotta et al., J Mol Neurosci 2011	Locomotor activity circadian rhythm Per2 expression	6-OHDA intraventricular injection Rats	Abnormal locomotor activity pattern in costant darkness with partial restoration in 12:12 LD
Vezoli J et al. Plos One 2011	Rest-activity rhythm (Non-parametric analysis) IS-IV-RA CR	MPTP monkeys (chronic low dose injection)	Early (before motor symptoms appearance), persistant and relevant fragmentation of rest-activity rhythm (increased IV and reduced IS) with reduced amplitude
Kudo et al. Experimental Neurology 2011	12:12 LD skeleton period (1h light + 11h darkness) DD Light exposure	Transgenic ASO mice (α-syn overxpression)	Disrupted daily and circadian rhythms in ASO mice, increasing with age Normal photic response in ASO mice Delayed sleep onset Normal Per2 expression in the SCN Reduced firing rate in the SCN

Table 6. Summary of studies on circadian rhythms findings in PD mouse models.

6. OPN4 POLYMORPHISM AND CHRONOTYPE

Chronotype or diurnal preference refers to the individual preference for timing of daily activities. Diurnal preferences represent an individual trait to be more active in the late evening (evening type or "owls") or in the morning (morning type or "larks").

This trait is normally distributed and can be assessed using questionnaires such as the Horne-Östberg Questionnaire (Horne and Östberg, 1976) or the Munich Chronotype Questionnaire (Zavada et al., 2005). The latter questionnaire assesses the habitual times of activity/sleep taking also into account the different behavior of the subject in free and workdays (Zavada et al., 2005). Both the questionnaires show a strong correlation with other phase markers of the circadian clock such as temperature nadir and melatonin peak time (Duffy et al., 2001).

Many studies have investigated the possible genetic basis of this individual trait mainly focusing on clock genes (*Per1*, *Per2*, *Per3*, *CLOCK* etc.) with frequently contrasting and non-reproducible results in different populations and cohort of individuals. The polymorphism in the clock genes most consistently associated with diurnal preference is the variable number of tandem repeats (rs57875989) in the coding region of the *Per3* gene (Archer et al., 2003). Indeed, individuals homozygotes for the longer variant ($PER3^{5/5}$) have a stronger morning preference than individuals homozygotes for the shorter variant ($PER3^{4/4}$) (Ellis et al., 2009; Archer et al., 2003). This genetic variant revealed the strongest correlation with other markers of the circadian phase, such as the phase of melatonin rhythm. Moreover, this polymorphism has been shown to affect also sleep homeostasis. In fact, individuals homozygotes for the longer variant ($PER3^{5/5}$) are characterized by more SWA during sleep and are more vulnerable to sleep loss in terms of cognitive functions than the individuals homozygotes for the shorter variant ($PER3^{4/4}$) (Viola et al., 2007). In fact, individuals homozygotes for the longer variant ($PER3^{5/5}$) showed greater reduction in fMRI-assessed brain responses to an executive task, in response to total sleep deprivation (Dijk and Archer, 2010). These findings ultimately suggest that higher homeostatic sleep pressure in $PER3^{5/5}$, together with the influence on the circadian pacemaker, modulates the amplitude of diurnal variation in performance, underlying the strict connection between circadian rhythmicity and sleep homeostasis. These findings were further consolidated by Schimdt and coauthors demonstrating by functional MRI that chronotype influences processing-related cerebral activity. In fact evening types, at difference with morning types, maintained or even increased their hemodynamic responses from the

subjective morning to the subjective evening, suggesting that morning types experience higher vulnerability to prolonged wake period (Schmidt et al., 2012).
Further, missense mutations in the *CK1δ* and *Per2* genes have been associated with advanced sleep phase syndrome (Wulff et al., 2009). A summary of main findings on genetic studies in relation to chronotype is provided in **Table 7** (modified from Steinmeyer et al., 2009).
To date, polymorphisms of the *OPN4* gene have been studied only in relation to SAD, a mood disorder with a strong circadian basis, characterized by the occurrence of depression in fall and winter (Roecklein et al., 2009). The authors found that one *OPN4* variant, the T/T genotype for the missense variant rs2675703 (P10L) may predispose some individuals to SAD. In fact, the T/T genotype was found only in the group of SAD patients and not in controls (Roecklein et al., 2009). The possible link between genetic polymorphisms of the *OPN4* gene and SAD is further reinforced by the finding that patients with SAD have an abnormal hypothalamic response to light, and in particular blue light, as documented by fMRI studies (Vandewalle et al., 2011b). Moreover, the prevalence of SAD and subsyndromal SAD is higher in delayed sleep phase syndrome individuals compared to controls, and, based on the role of mRGCs in the regulation of circadian rhythms, it is possible to hypothisize a possible role of genetic variants in the *OPN4* gene in the determination of the circadian phenotype (Lee et al., 2011).

Table 7. Summary of genetic studies on chronotype (modified from Stenmeyer et al., 2009)

Gene	Species	Region	SNP	AA change	Phenotype	Association	Publication
CLOCK	Human	Exon 17	A1982G	Yes	DSPS and N-24	No	Iwase et al. (2002)
		Exon 17	G1955A	Yes	DSPS and N-24	No	Iwase et al. (2002)
		3' UTR	T3111C	No	Morningness–eveningness	Yes	Mishima et al. (2005)
				No	Morningness–eveningness	Yes	Katzenberg et al. (1998)
				No	Morningness–eveningness, DSPS	No	Robilliard et al., (2002)
				No	DSPS and N-24	No	Iwase et al. (2002)
				No	Morningness–eveningness, DSPS	No	Pedrazzoli et al. (2007)
		5' UTR	T257G	No	Morningness–eveningness, DSPS	No	Pedrazzoli et al. (2007)
PERIOD1	Human	Exon 18	T2434C	No	Morningness–eveningness	Yes	Carpen et al. (2006)
		Exon 18	A2548G	No	Morningness–eveningness	No	Katzenberg et al. (1999)
PERIOD2	Human	Exon 17	A2106G	Yes	ASPS	Yes	Toh et al. (2001)
		Promotor	C-1228T	No	Morningness–eveningness	No	Carpen et al. (2005)
		5' UTR	C111G	No	Morningness–eveningness	Yes	Carpen et al. (2005)
		Exon 23	G3853A	Yes	Morningness–eveningness	No	Carpen et al. (2005)
PERIOD3	Human	Exon 15,17, 18, 20	T1940G, C2590G, T3110C,A3473A, del(3031-3084nt)	Yes	DSPS	Yes	Ebisawa et al. (2001)
		Exon 18	4-/5-repeat		Morningness–eveningness	Yes	Jones et al. (2007)
			del(3031–3084nt)		Morningness–eveningness, DSPS	Yes	Archer et al. (2003)
		Exon 15	T1940G	Yes	Morningness–eveningness	Yes	Johansson et al. (2003)
AANATc	Human	Promotor	T-542G	No	Sleep pattern	No	Wang et al. (2004)
		Promotor	G-263C	No	Sleep pattern	Yes	Wang et al. (2004)
		Exon 4	C702T	No	DSPS	No	Hohjoh et al. (2003)
		Exon 4	C756T	No	DSPS	No	Hohjoh et al. (2003)
		Exon 4	G619A	Yes	DSPS	yes	Hohjoh et al. (2003)
CKId	Human	Exon 2	T44Ab	Yes	FASPS	Yes	Xu et al. (2005)
CKIe	Human	Exon 9	G1223A	Yes	DSPS and N-24	Yes	Takano et al. (2004)
MTNR1a	Human	Exon 1	C160T	Yes	DSPS and N-24	No	Ebisawa et al. (1999)
		Exon 2	C470T	Yes	DSPS and N-24	No	Ebisawa et al. (1999)
MTNR1b	Human	Exon 1	G71A	Yes	DSPS and N-24	No	Ebisawa et al. (2000)
		Exon 1	C196T	Yes	DSPS and N-24	No	Ebisawa et al. (2000)
TIMELESS	Human	Exon 20	A2634G	Yes	Morningness–eveningness	No	Pedrazzoli et al. (2000)
HLA DR1	Human				DSPS	No	Hohjoh et al. (1999)

AIMS

Our previous studies on the mRGCs system in Leber's hereditary optic neuropathy, a neurodegenerative disease selectively affecting RGCs, have shown relative sparing of mRGCs and maintenance of the melanopsin-mediated melatonin suppression response to light. This observation explained the maintenance of pupillary light reflex (PLR) in these patients and pointed to the question of how mRGCs may be resistant to neurodegeneration. Thus, we wanted to verify if this is the case in other neurodegenerative diseases affecting RGCs, such as dominant optic atrophy. Further, we developed the hypothesis that mRGCs system could be affected and responsible, at least in part, for the occurrence of circadian rhythms disturbances in common neurodegenerative disorders such as Alzheimer disease, Parkinson disease and ultimately in normal human aging, based on the common feature of optic neuropathy and RGCs loss in all these conditions. Lastly, we hypothesized that functional genetic variation in the *OPN4* gene, encoding for the melanopsin photopigment, may influence human chronotypes.

Based on these premises this PhD thesis is articulated in three sections with the following aims:

- extend the characterization of the mRGCs system in blind patients with hereditary optic neuropathies (Project 1)
- explore the role of mRGCs system in age-related circadian abnormalities and the possible involvement of these cells in neurodegenerative disorders with evidence of retinal and/or circadian abnormalities (Parkinson, MCI and Alzheimer) (Project 2)
- investigate if genetic variation in the melanopsin gene may be associated to morningness/eveningness phenotypes (Project 3)

Concerning the 1st aim of this project we investigated:

1. the mRGCs system in dominant optic atropy
2. the mRGCs contribution to PLR in LHON patients by pupillographic recordings
3. the role of the photopigment melanopsin in mRGCs resistance to neurodegeneration

Concerning the 2nd aim of this project we investigated:

1. the occurrence of optic neuropathy in AD, MCI and PD patients
2. the occurrence of rest-activity circadian rhythm dysfunction in AD, MCI and PD patients

3. the pathology of mRGCs in post-mortem retinal and optic nerve specimens from AD patients and age-matched controls
4. the possible correlation between the occurrence of circadian dysfunction and optic nerve involvement in AD and PD

Concerning the 3rd aim of this project we investigated:

1. the association of the P10L *OPN4* missense polymorphic variant with human chronotypes
2. the association of the P10L *OPN4* missense polymorphic variant with seasonal affective disorder

METHODS

1. PATIENTS

Project 1

1.1. *Immunohistochemical analysis of mRGCs and axonal count in optic nerve cross-sections of a dominant optic atrophy case and age-matched control*

We performed immunohistochemical analysis of mRGCs and axonal counts in a 87-year-old dominant optic atrophy patient and a 85-year-old control case.

1.2. *Pupillometric evaluations in LHON*

Pupillometric evaluation was performed in:

- 13 LHON patients (mean age 44.8 ± 17.5; disease duration 18.2 ± 10.3 years) (10 males and 3 females)
- 16 age-matched controls (mean age 43.4 ± 14.9; 7 males and 9 females)

For pupillometric evaluation only one eye was tested for each subject. The eye with the worst visual acuity was tested in LHON patients.

Project 2

2.1. *In-vivo evaluation of AD, MCI and PD patients by OCT, actigraphic recordings and sleep questionnaires*

All participants gave their informed consent to this prospective study, which was approved by the Internal Review Board (Comitato Etico Indipendente, Azienda Unità Sanitaria Locale di Bologna, Protocol #656/CE).

Inclusion criteria for patients were:

1. Diagnosis of Alzheimer disease according to NINCDS-ADRDA criteria, (McKhann et al., 1984). Patients with mild-moderate disease severity were included (Mini Mental State examination-MMSE score between 11 and 26)
2. Diagnosis of mild cognitive impairment (Petersen et al., 2001)
3. Diagnosis of probable Parkinson disease according to Gelb Criteria (Gelb et al., 1999)

Exclusion criteria for patients were:

1. Spherical or cylindrical refractive errors higher than 3 or 2 diopters respectively
2. Posterior pole pathology such as macular degeneration and optic neuropathies including glaucoma
3. Unsustained fixation due to rest tremor
4. Severe AD (MMSE <11)

Inclusion criteria for controls were the absence of cognitive dysfunction as confirmed by MMSE and Brief Battery for Mental Deterioration, and absence of ocular pathologies as confirmed by ophthalmologic evaluation.

Optical coherence tomography (OCT) evaluation was performed in:

- 20 AD patients (age 72.2 ± 10.6; range: 51-84 years; 11 males and 9 females)
- 11 MCI patients (age 73.1 ± 6.2; range: 64-87 years; 6 males and 5 females)
- 43 PD patients (age 65.6 ± 8.4; range: 46-83 years; 23 males and 20 females)
- 88 controls (age 65.7 ± 10.7; range: 46-85 years; 36 males and 52 females)

One-week actigraphic monitoring (Actigraph Mini Motion Logger, Ambulatory Monitoring Inc) was performed in:

- 15 AD patients (age 70.9 ± 13.3; range: 52-84 years; 8 males and 7 females)
- 7 MCI patients (age 72.4 ± 5.1; range: 64-78 years; 5 males and 2 females)
- 9 PD patients (age 64.3 ± 8.2; range: 50-76 years; 4 males and 5 females)
- 10 controls (age 65.8 ± 7.5; range: 54-80 years; 5 males and 5 females)

2.2. *Immunohistochemical analysis of mRGCs and axonal count in optic nerve cross-sections in post-mortem specimens from AD patients and controls*

We have been able to collect and analyze:

- 14 neuropathologically confirmed AD cases (age 82.6 ± 12.3; range: 62-98 years; 8 males; 6 females)
- 11 controls (age 78.5 ± 15.1; range 58-105)

The inclusion criterium for AD cases was a neuropathological diagnosis of definite AD (Braak stage V or VI), according to modified CERAD criteria (Mirra et al., 1991) and NIA/Reagan criteria (Hyman & Trojanowski, 1997)

Project 3

The genetic analysis of the single nucleotide polymorphism (rs2675703-P10L-exon1) in the *OPN4* gene was performed in 314 subjects (age 31.8 ± 9.9; range: 20-73; 103 males and 211 females), classified for chronotypes according to the Morningness-Eveningness questionnaire (MEQ) (Horne and Östberg, 1976). In a subgroup of subjects (n=182) we also retrieved SPAQ questionnaires assessing the presence of Seasonal Affective Disorder syndrome and sub-syndrome (Rosenthal et al., 1984; Muscettola et al., 1995; Magnusson et al., 1996).

2. IN-VIVO STUDIES

2.1. Pupillometry

For pupillometric evaluation of the pupillary light reflex (PLR) in LHON patients belonging to a large Brazilian pedigree (Sadun et al., 2003) and controls we used the same protocol published by Park and coauthors (Park et al., 2011). The setting and the general scheme of the pupillometric protocol are depicted in **Figure 11**.

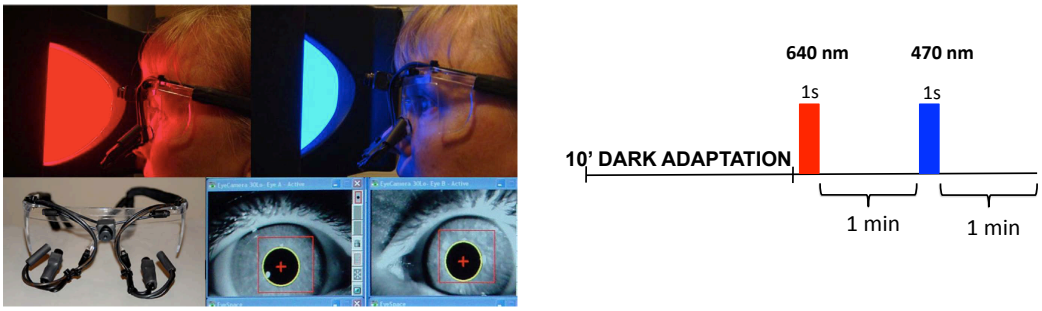


Figure 11. LED-driven Ganzfeld system and binocular eye-tracking camera (left) (Park et al., 2011); General scheme of the pupillometric evaluation (right)

Briefly, we used alternating 1 sec blue and red light flashes at increasing intensities (from 1cd/m2 to 250 cd/m2) with different interstimulus durations, after 10 minutes of dark adaptation, as detailed below:

1. RED0 (10''-30'')= 1cd/m2
2. BLUE0 (10''-30'')=1cd/m2
3. RED1 (10''-30'')=10cd/m2
4. BLUE1 (10''-50'')=10cd/m2
5. RED2 (10''-1'10'')=100cd/m2
6. BLUE2 (10''-1'10'')=100cd/m2
7. RED 2.4 (10''-1'10'')=250cd/m2
8. BLUE 2.4 (10''-1'10'')=250cd/m2

This protocol allows at isolating the contribution of rods, cones and mRGCs to PLR response. Light stimulation in all experiments was controlled by a LED-driven Ganzfeld system (*Super Color Ganzfeld Q450 SC, Roland Consult, Germany*). This system generates a wide range of stimuli from -4 to 2.6 log cd/m2 for blue (467 ± 17 nm) lights and from -4 to -2.6 log cd/m2 for red (640 ± 10 nm) lights.
A binocular eye-tracking camera system with infrared illuminating diodes (*Arrington Research, Scottsdale, AZ*) was used for real-time pupil recording with a sample rate of 60 Hz. The miniature video camera system is attached to a plastic eye frame so that there is no physical contact between the camera and the eye and so that a wide visual field view is afforded.

The Rod-driven PLR is best studied at 1-sec low intensity (lower than -1 log cd/m2) blue-light stimuli in the dark. The Melanopsin-driven PLR is best studied at 1-sec high intensity (2.6 log cd/m2) blue-light stimuli in the dark. The cone-driven PLR is best studied at 1-sec red flash on blue background, which suppresses rod and melanopsin response.

The PLR to red stimuli shows a monotonic increase, a saturation of peak amplitudes at high intensities and a fast recovery. The PLR to blue stimuli is characterized by the emergence of a sustained response after the 1-sec stimuli for intensities of 1 log cd/m2 or greater (**Figure 12**)

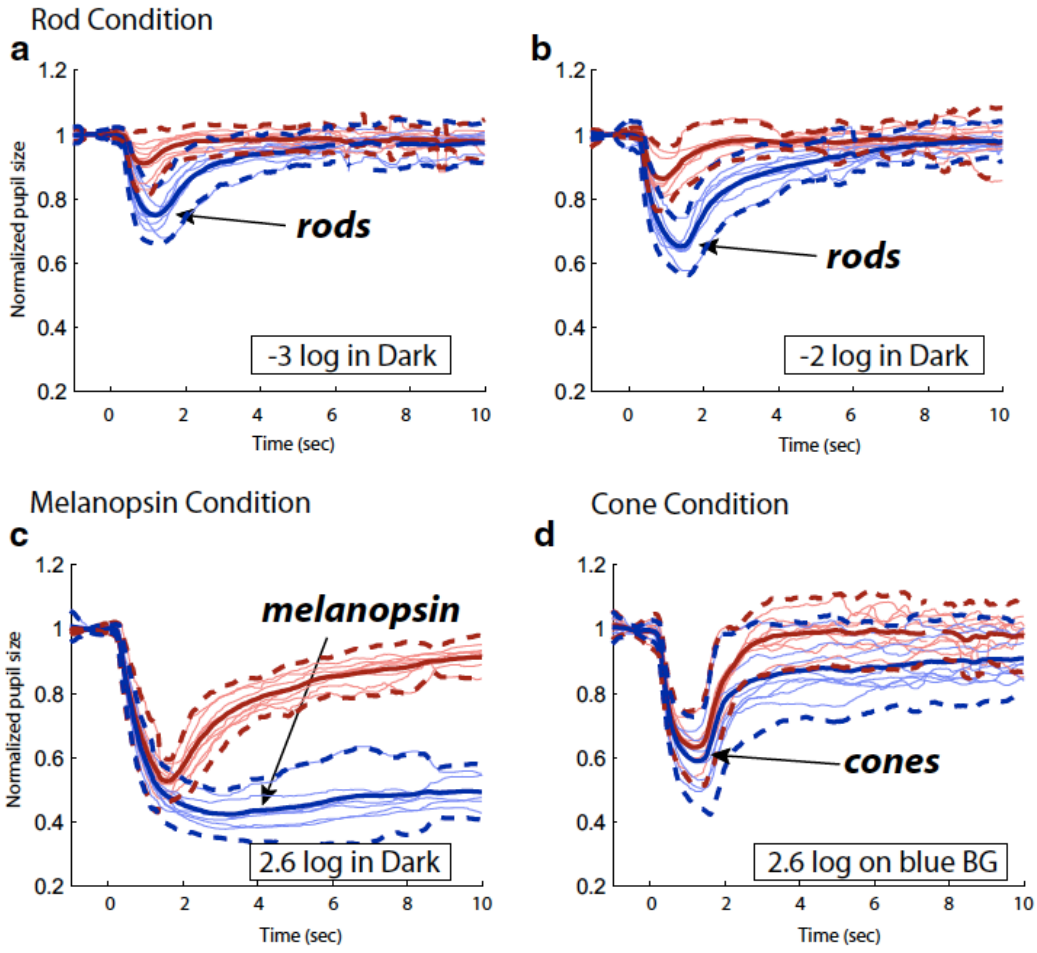


Figure 12. Representation of the experimental condition for the isolation of the contribution of the single photoreceptors to the pupillary light reflex (upper panels: rod condition; left lower panel: melanopsin condition; right lower panel: cones condition)

In order to compare inter-individual responses, raw pupil size curves have been transformed using the normalized measures relative to the individual baseline value.

For each condition (blue and red light) and each individual, two variables were computed: peak amplitude (PA) and sustained response (SR) (**Figure 13**).

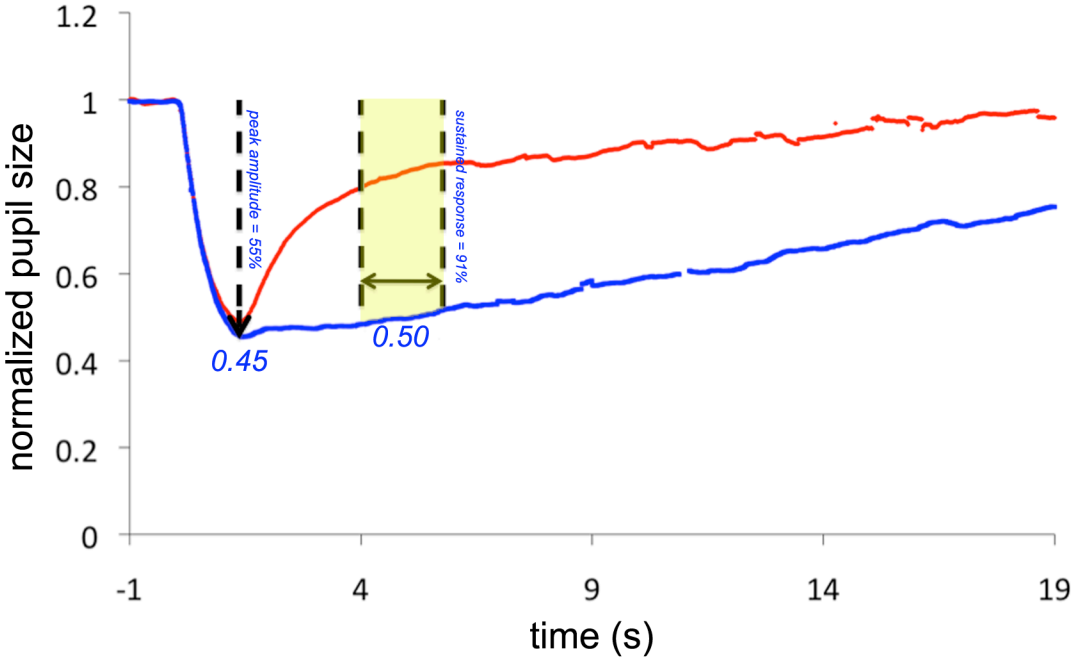


Figure 13. PA is the absolute value of the normalized pupil size variation, expressed as a percentage of variation from baseline. SR is the median value between 4 and 6 seconds after light onset, computed similarly to PA, and expressed as percentage of PA

A severity score of pupillary light response was computed assessing the number of pathological parameters (below 2SD from the mean of controls).

Parametric t-test comparisons were used to assess the differences between patients and controls ($p < 0.05$). Pearson test was used to assess the correlation between the pupillometric parameters, age, disease duration and visual acuity ($p<0.05$).

2.2. Optical coherence tomography

All subjects underwent RNFL thickness measurement by OCT (*StratusOCT, software version 4.0.1; Carl Zeiss Meditec, Inc, Dublin, CA*) (**Figure 14**). We used the RNFL thickness 3.4 acquisition protocol (Barboni et al., 2005, 2010). For each eye, we studied the average RNFL thickness (360-degree measure), as well as temporal (316–45-degree unit circle), superior (46–135 degrees), nasal (136–225 degrees), and inferior quadrant thickness (226–315 degrees.) At the beginning of the examination, the OCT lenses were adjusted for the patient's refractive error. Polarization was optimized to maximize the reflective signal, and the best centration of the scan with respect to the optic disc was used.

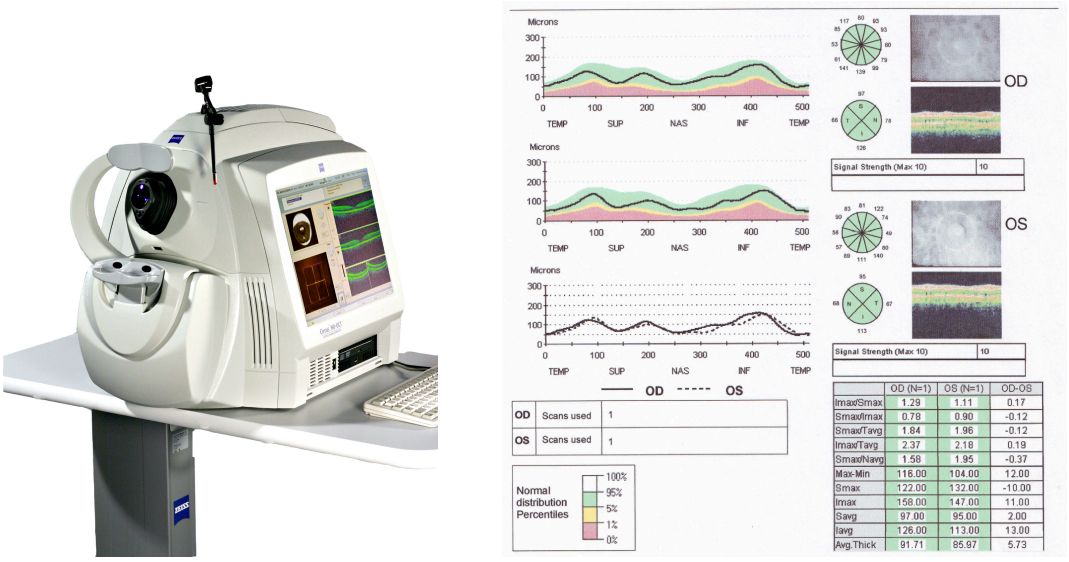


Figure 14. Stratus OCT (left); Example of RNFL thickness 3.4 acquisition protocol for a control subject. RNFL thickness for each quadrant is provided for OD and OS (right)

For the statistical analysis one eye for each control, AD and MCI patient, was randomly chosen, having verified that there was no statistical difference between right and left eye, as previously reported (Barboni et al., 2005). Based on the characteristic asymmetry of PD we analyzed both the eyes homolateral and contralateral to the most affected body side in PD patients.

Normal distribution of RNFL thickness measurements was evaluated by Kolmogorov-Smirnov test. Differences between patients and controls were assessed by ANCOVA using age and gender as covariates, followed by post-hoc pairwise comparisons. Significance was assumed for $p<0.05$. For PD patients the significance was corrected for multiple comparisons according to the Bonferroni method. Correlation bivariate analysis was performed using Pearson coefficient ($p<0.05$).

For PD patients multivariate analysis was performed to investigate the effect of genetic, demographic (age and gender) and clinical parameters (age at onset, disease duration, UPDRS-III score and stage of disease) on RNFL measurements using a multiple regression with a backward stepwise method to obtain a significant model in which all included variables had a $p<0.05$. The same analysis was performed for AD and MCI patients including demographic (age and gender) and clinical parameters (age at onset, disease duration and MMSE score). AD patients were further analyzed by subgrouping them, according to the MMSE score, in mild (≥ 20) and moderate (≤ 19) cases (Folstein et al., 1975).

2.3. Actigraphic recordings

Spontaneous motor activity was measured by a wrist actigraph (*Actigraph Mini Motionlogger, Ambulatory Monitoring Inc. Ardsley, NY, USA*) worn on the non-dominant wrist for 7 days. In PD patients actigraph was placed on the least affected side for unilateral cases and on non-dominant wrist for bilateral cases. Data were collected in 60-second epochs in 24-hour periods. An example of actigraphic monitoring for 7 days is provided in **Figure 15**.

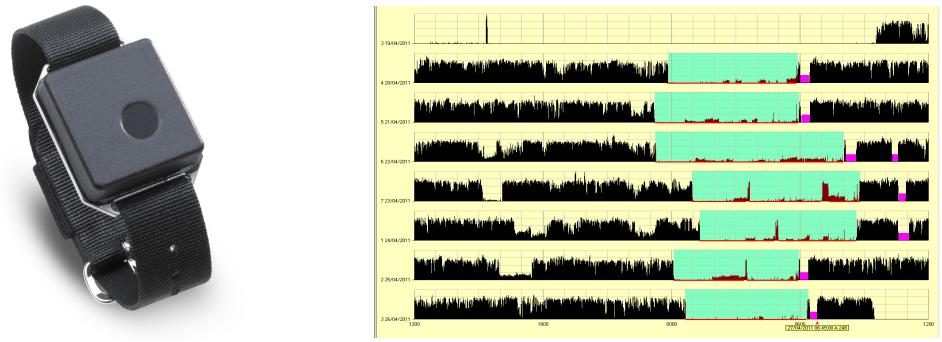


Figure 15. Example of actigraph (left); example of actigraphic monitoring for 7 days in a control subject (right).

The collected data were analyzed by a commercial software (*AW2, Ambulatory Monitoring Inc.*), which allowed retrieving the following parameters:

1. sleep minutes (minutes of rest activity during the night period)
2. sleep efficiency (sleep time divided by the time in bed multiplied by 100).

Each participant also completed a sleep diary over the seven days of monitoring indicating bed/rise time, time to sleep onset and eventual daytime naps.

Raw actigraphic data were firstly inspected in order to detect the correspondence with the subject sleep diary and eventual artifacts (low activity due to the actigraph removal). Each single artifactual epoch was marked as "bad" epoch. Furthermore, to allow the comparison between subjects, all the recordings were aligned with the starting time at 12:00 am. Given that five subjects wore the actigraph for less than 7 days (three for six days and two for five days), in order to adequately compared subjects, consecutive 120 hrs (5 days) of recordings were considered for subsequent analysis. Data were then exported to Matlab and the mean of each hour was computed as the mean of the "good" epochs. If consecutive "bad" epochs occupied an entire hour, these periods were treated as missing data and the hourly mean was calculated as the weighted mean of the pre and post-activity counts (**Figure 16**).

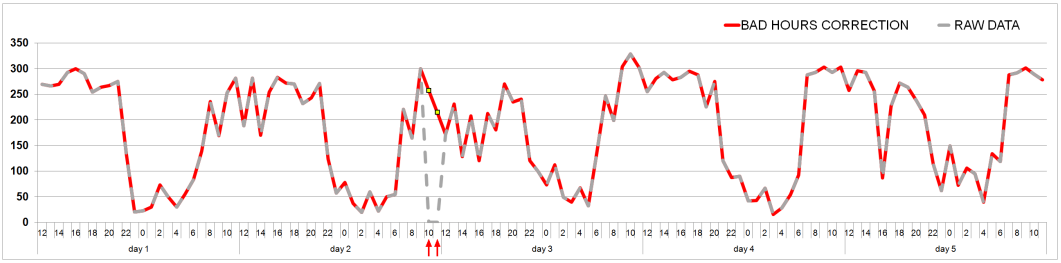


Figure 16. Five-days profile of hourly mean activity values in a subject. In the original data (grey dashed line) values of two consecutive hours are lacking (red arrows) due to the absence of artifact-free epochs in the entire 120' interval. The Red line represent the activity profile after the replacement of the missing data. The new values (yellow squares) were computed as the weighted mean of adjacents valid data.

For the analysis of circadian parameters derived from actigraphic recordings we chose to apply non-parametric methods (Witting et al., 1990; Van Someren et al., 1997a,b; Van Someren et al., 1999) because the rest-activity rhythm is highly non-sinusoidal and thus not well described using a cosinor analysis (Van Someren et al., 1999).

For all subjects the following non-parametric measurements were computed:

- Interdaily stability: **IS (0-1):** quantitative measure of the invariability of the rhythm between the days (index of the strength of the rhythm to supposedly stable enviromental zeitgebers). Higher values indicate a more stable rhythm. IS is calculated as the ratio between variance of the average 24-hour pattern around the mean and the overall variance.

$$IS = \frac{n \sum_{h=1}^{p} (\overline{x_h} - \overline{x})^2}{p \sum_{i=1}^{n} (x_i - \overline{x})^2}$$

- Intradaily variabily: **IV (0-2):** indication of the fragmentation of the rhythm (i.e. frequency and extent of transitions between rest and activity). IV is calculated as the ratio of the mean squares of the difference between successive hours (first derivative) and the mean squares around the grand mean (overall variance)

$$IV = \frac{n \sum_{i=2}^{n} (x_i - x_{i-1})^2}{(n-1) \sum_{i=1}^{n} (\overline{x} - x_i)^2}$$

N=total number of data;p=number of data per day; $\overline{x}$=mean of all data; $\overline{x_h}$ = hourly means; x_i= individual data points

- Relative amplitude: normalized difference between the most active 10h period (M10) and the least active 5h period (L5) in the average 24h pattern. Higher values indicate a stronger rhythm.

$$RA = \frac{M10 - L5}{M10 + L5}$$

L5: sequence of the least-active hours in the 24-hour average activity profile. Average activity during L5 provides an indication of trough or nadir of the rhythm (i.e. regularity and restfulness of sleep period). Lower value indicate more restful sleep.
M10: sequence of the 10 most-active hours in the 24-hour average activity profile. Average activity during M10 provides an indication of the peak of the rhythm (how active and regular the wake periods are).

For PD patients levodopa-equivalent daily dose (LEDD) was calculated (100 mg L-dopa equivalent to 1 mg cabergoline/lisuride/pergolide/pramipexole, 5 mg ropinirole, 10 mg apomorphine, 76,92 mg L-dopa plus COMT inhibitor or 133,3 slow release L-dopa formulation) (Wenzelburger et al., 2002).

Differences in actigraphic parameters between patients and controls were assessed by parametric unpaired t-test ($p<0.05$). Correlation bivariate analysis between actigraphic data, demographic (age, gender) and clinical parameters (UPDRS-III, H&Y stage of disease, and LEDD for PD; MMSE score for AD and MCI; disease duration and OCT measurements for PD, AD and MCI) was performed using Pearson coefficient ($p<0.05$). We also stratified AD patients by MMSE score in two groups, mild (MMSE$\geq$20) and moderate (MMSE$\leq$19) and differences between the groups were assessed by unpaired t-test ($p<0.05$). For the analysis at single subject level we computed z-scores for all the actigraphic parameters investigated based on mean and SD of controls. Patients with more than 2 SD difference from the mean of controls were considered “circadian impaired”. Differences in clinical variables between “circadian impaired” and “circadian unimpaired” subgroups were evaluated by means of unpaired t-test ($p<0.05$). Correlations between clinical and circadian variables were computed separately for the two subgroups.

2.4. Sleep questionnaires

To investigate the occurrence of sleep disturbances we also included self-administered questionnaires.
Sleep questionnaires included:
1. Epworth Sleepiness Scale (Vignatelli et al., 2003). The score range is 0-24 with a score of 10 or more indicating excessive daytime sleepiness
2. Pittsburgh Sleep Quality Index (Buysse et al., 1989) with a score more than 5 indicating pathological subjective sleep quality
3. Berlin Questionnaire (Netzer et al., 1999) assessing the risk of sleep apnea
4. Parkinson disease sleep scale (Chauduri et al., 2002)

The Parkinson disease sleep scale consists of 15 items addressing at the following items: overall quality of night's sleep (item 1), sleep onset and maintenance insomnia (items 2 and 3), nocturnal restlessness (items 4 and 5), nocturnal psychosis (items 6 and 7), nocturia (items 8 and 9), nocturnal motor symptoms (items 10–13), sleep refreshment (item 14) and daytime dozing (item 15). Scores for each item range from 0 (symptom severe and always experienced) to 10 (symptom-free). The maximum cumulative score for the PDSS is 150 (patient is free of all symptoms).
Differences between patients and controls were assessed by unpaired t-test ($p<0.05$).

Neurological examination was performed in all patients included in the study.
Disease severity (UPDRS-III score-off, Hohen &Yahr stage of disease-off), disease duration, concomitant disease and therapy were retrieved for all patients
Cognitive functions were assessed in AD, MCI patients and controls by Mini Mental State Examination (MMSE) score corrected for scholarity (Folstein et al., 1975), and if possible with Brief Battery for Mental Deterioration (BBDM) (Gallassi et al., 1986, 2002)

3. IN-VITRO STUDIES

3.1. Melanopsin expression in Hek cells at different stressful paradigms and light conditions

To test if the melanopsin expression influences cell viability in different stressful paradigms, under different light conditions (darkness, blue and white light), we used a tumor-derived immortal cell line (HEK-hMel-1) with stable expression of melanopsin under the control of a tetracycline-sensitive promoter. Melanopsin-expressing cells have been tested in darkness and in different light conditions in comparison with the same cells not expressing melanopsin, after pro-oxidant challenge with tert-butyl-hydroperoxide (TBH) or rotenone. Rotenone is a respiratory complex I inhibitor which induced ROS production whithin mitochondria simulating the pathological condition determined by the LHON mtDNA mutations. TBH induces a cytoplasmic increase of ROS, representing a different paradigm of ROS exposure. Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 100μg/ml Hygromicin B, 15μg/ml Blasticidin, in an incubator with a humidified atmosphere of 5% CO_2 at 37 °C.

Tetracycline (1μg/ml) was added to promote melanopsin expression, which was verified by Western Blot. We seeded 100.000 cells in each well (24 well plates) and cell viability was detected by the sulforhodamine B (SRB) absorbance at 570 nm with a VICTOR3 Multilabel Plate Counter as previously described (Porcelli et al., 2008). Stress paradigms were applied exposing cells to increasing concentrations of rotenone (0.5 μM; 2.5 μM; 5 μM) and TBH (50 μM; 200 μM). The experiments were performed in different light conditions, which included darkness, daylight and 30 minutes of exposure to blue and white light. Experiments were performed with and without retinal (5 μM), a co-activating factor for melanopsin photoreception.

4. POST-MORTEM STUDIES

4.1. Immunohistochemical analysis of melanopsin-containing retinal ganglion cells in human post-mortem retinas

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 collotes containing the entire retina (nasal and temporal), at that level bisecting the papillomacular bundle (**Figure 17**).

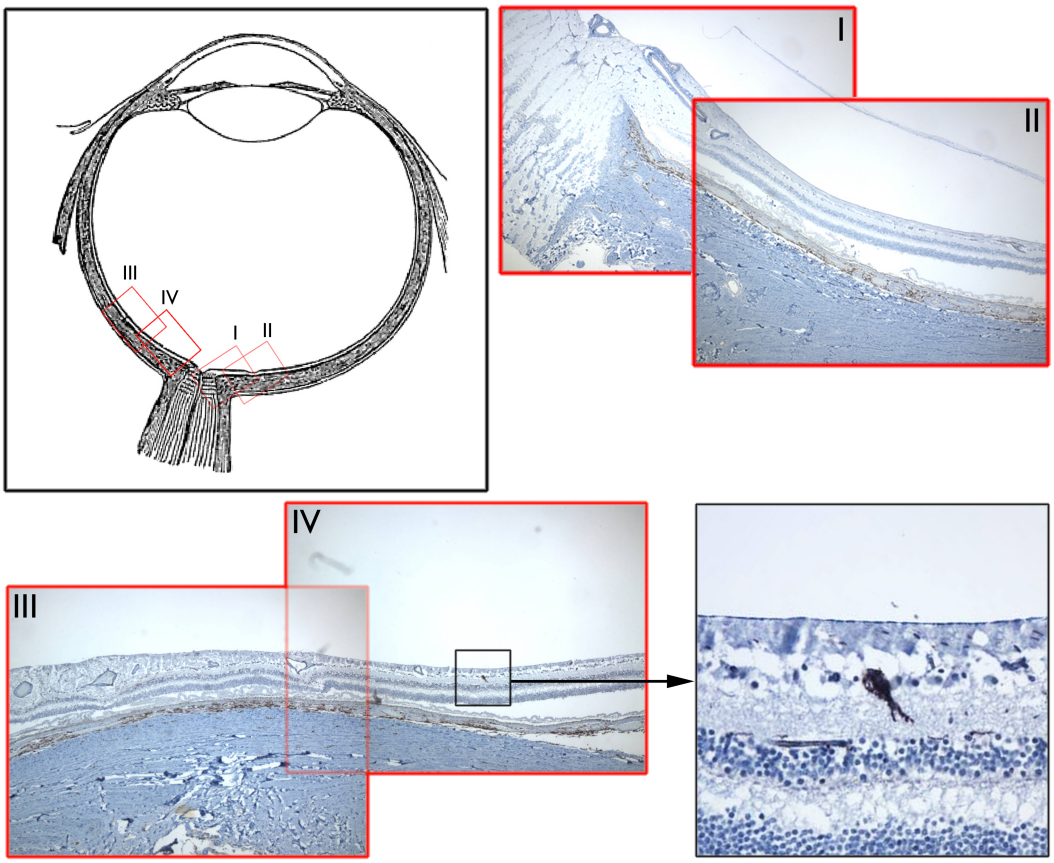


Figure 17. Upper left. Schematic representation of the horizontal section passing through the macula and the optic nerve head. An example of retinal reconstruction is provided (red rectangles). Upper right. Higher magnification pictures of the I and II captures (5X); optic nerve head (I) and retinal layers are visible (II). Lower panel left. An other example of embricated captures at 5X is provided (III and IV). Lower panel right. Higher magnification detail of mRGC in the RGL from the IV caption is provided.

Tissue from the superior half was embedded in paraffin and serially sectioned at 5 mm. Sections were immunostained for melanopsin [rabbit anti-melanopsin, code no.

5J68, characterized in detail by Hannibal et al., 2004a]. We used an indirect immunoperoxidase technique with diaminobenzidine as the substrate/chromogen (Hannibal et al., 2004a). For each subject we analyzed one eye both for the mRGCs and axonal count. 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 (**Figure 18**).

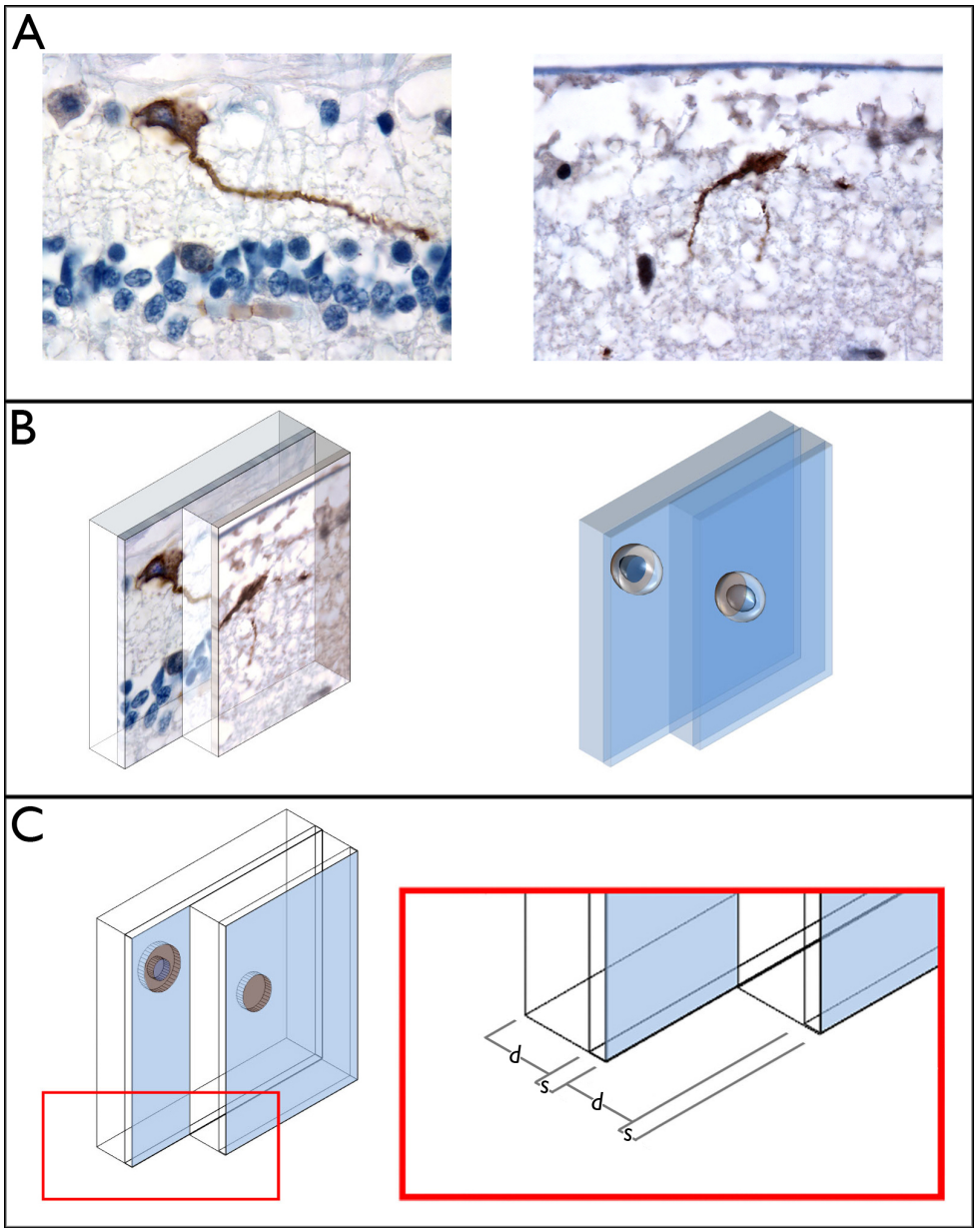


Figure 18. (A) On the left one example of mRGCs with nucleus and cytoplasm visible; on the right a fragment of cytoplasm is shown. (B) Tridimensional and schematic reconstruction of the mRGC cut through the nucleus and peripherally (C) Schematic representation of the single sections (width: 5 microns) and the distance between two sections (d=20 microns).

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 (**Figure 17**). Counting of mRGCs allowed for calculation of their area density across the posterior retina in the nasal/temporal axis. This retinal cross-section, 5 mm 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 mm) plus the thickness of the retinal section (5 mm), 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 mm). The total retinal area density of mRGCs in each eye was calculated assuming an average total surface in human retina of 1040mm2 and an even superior/inferior distribution of mRGCs (Curcio and Allen, 1990). Total RGC number was calculated by assessing the number of axons in the optic nerve under the assumption that one RGC produces one axon. Thus, the total number of RGCs was derived by counting the axons in the optic nerve cross-sections using paraphenylenediamine (PPD)-based manual count (see next section). Finally, we calculated the ratio between the mRGCs density and total RGCs.

4.2. Morphometric assessment of axonal population in optic nerve cross-sections

Optic nerves were cut into cross-sections 2mm thick and 3mm posterior to the globe. Orientation was established by razor nicks and the specimens were processed for paraffin and plastic blocks. Semi-thin sections were cut on an ultramicrotome at 1 mm from plastic-embedded tissues and stained with paraphenylenediamine for light microscopic examination of myelin profiles (Tenhula et al., 1992). Only for the DOA case the paraffin tissue blocks were cut with a microtome at 5 mm, stained with haematoxylin and eosin and immunostained using an indirect method with horseradish peroxidase and diaminobenzidine as substrate/chromogen for neurofilaments. 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 was manually performed on images acquired at 1000X with a 100X oil immersion lens. For total axon counts, each nerve cross section was partitioned into 8 regions (temporal, nasal, superior, inferior, and a set of inner/outer for each). Four pictures for each region were taken under 1000X

magnification to generate 32 zones. Counts were done by hand and confirmed by two independent observers. Cross sectional areas were measured for each region using the "Spot II Advanced" software package. Total axons counts and axon densities were then calculated for each region and extrapolated for the entire optic nerve.

4.3. Neuropathological scoring of AD brain pathology

A recently proposed "ABC" score was applied to grade the severity of brain pathology of AD patients included in this study (Montine et al., 2012). We were able to compute only the "B" and the "C" scores using the silver stain for the detection of neurofibrillary tangles ("B") and the thioflavin stain for the detection of neuritic plaques ("C"), respectively. The brain regions analyzed were: hippocampus CA-1 (uncus and lateral geniculate body), entorhinal cortex, middle frontal, superior/middle temporal, inferior parietal, primary visual and visual associative cortex.

4.4. Counts and statistical analysis

For each retina we retrieved:
1) total number of mRGCs
2) density of mRGCs
3) axonal count
4) mRGCs/RGCs ratio

Unpaired t-test was used for comparison between groups. We also stratified both controls and AD patients by age, using the median age (80 years) as cut-off. We used ANOVA analysis for comparison between groups with Bonferroni post-hoc correction for multiple comparison ($p<0.05$). Correlations between mRGCs density, total axonal counts, mRGCs/RGCs ratio, age, Braak stage and neuropathological scores were assessed by Spearman test ($p<0.05$).

5. GENETIC STUDIES

Association study of a polymorphic variant in the OPN4 gene with human chronotypes and seasonal affective disorder

We assessed the missense variant rs2675703 (P10L) in the melanopsin gene (*OPN4*), previously reported by Roecklein and coauthors as associated with the Seasonal Affective Disorder (Roecklein et al., 2009). This SNP was previously identified and reported through the NCBI dbSNP public database (Sherry et al., 2001) (**Figure 19**). Reported frequencies in caucasian of the three genotypes of the P10L variant in caucasian are 73% (CC), 25% (TC) and 2% (TT).

Single nucleotide polymorphisms markers, PCR primers, and probes

RefSNP[a]	Location[b]	bp gap[c]	PCR primers (F: forward; R: reverse)	MGB® probes (3' to 5')[d]	Failure rate (%)	Error rate (%)
rs2675693	Flanking 5'	3118	F: 3'-AAGAAAGACCTCAGGCCCCTAT-5' R: 3'-CCTGAGCCTCAGTTTCTCCAA-5'	Fam: 3'-AGGCAAGGCCGGGG-5' Vic: 3'-AGATAGATCAAAGCCCA-5'	<1 <1%	0
rs2675703 (P10L)[e]	Exon 1	1	F: 3'-AGGAAAGTTGGGAGGCTGAG-5' R: 3'-GGTCAGGGAAGGCTCTGTG-5'	n/a	7.6	0
rs11202106	Exon 1	3154	(Sequenced with rs2675703/P10L primers)	n/a	4.4	0
rs10788521	Intron (2)	1917	F: 3'-CACGTGTGTGCACATGCATAC-5' R: 3'-TCCTGACACCTCCAGAGCTATG-5'	Fam: 3'-TGAGGGGTGCGGGA-5' Vic: 3'-TGAGGGGTGTGGGAA-5'	<1	0
rs3740335	Intron (5)	2475	F: 3'-GCTGACTGCCACCCGACTA-5' R: 3'-AAGGTCTGGAGAGCCCTAGGA-5'	Fam: 3'-TGGACGATGCGTCCT-5' Vic: 3'-TGGACGATGCATCCT-5'	<1	0
rs1079610 (I394T)[e]	Exon 8	1376	F: 3'-TGGCTTCTTCCCCCAGTGTA-5' R: 3'-ATGTGCTTGGTGTGCATTCAG-5'	Fam: 3'-CTCCACGCTGATCAG-5' Vic: 3'-CTCCACGCTGACCAG-5'	4.9	9
rs12262894 (D444G)[e]	Exon 9	2550	F: 3'-TGCCCAGCAAGCAAATGG-5' R: 3'-GGTGCCTTGGCTTCCAAGTC-5'	Fam: 3'-TACGGTCAGGATCTG-5' Vic: 3'-TACGGTCAGGGTCTG-5'	<1	0
rs3740341	3' UTR	2293	F: 3'-TGGCTTCTTCCCCCAGTGTA-5' R: 3'-ATGTGCTTGGTGTGCATTCAG-5'	Fam: 3'-TCTTGTGCACGCGGG-5' Vic: 3'-TCTTGTGCACACGGG-5'	<1	0
rs2803558	3' Flanking	n/a	F: 3'-CAGCTATTCTTAGGAGCCTCTCAAGAG-5' R: 3'-AAAGGGTAGAGAGGGTTGAGTGAGA-5'	Fam: 3'-AGGCAAGGCCGGGG-5' Vic: 3'-AGGCAAGGCTGGGG-5'	<1	0

Figure 19. Details of the P10L SNP are reported (Roecklein et al., 2009)

Chronotypes were assessed by the Horne–Ostberg morningness-eveningness (ME) Italian questionnaire (MEQ) to assess the ME preferences (Horne & Ostberg, 1976). Chronotypes were classified according to the MEQ into: extreme morning (70-86), moderated morning (59-69), intermediate (42-58), moderate evening (31-41), extreme evening (16-30). The analysis was also performed grouping the extreme and moderate morning and the extreme and moderate evening in two single categories.
To assess the occurrence of the seasonal affective syndrome or subsyndrome we used the validated Italian Version of the SPAQ questionnaire (Muscettola et al., 1995). This questionnaire is composed by three different sections. One of these, the Seasonality Score Index (SSI) or Global Seasonality Score (GSS), has six items that measure seasonal variations in mood, appetite, weight, sleep, energy and socialising. The sum of these items is the Seasonality Score (SS), which can range from 0 to 24. Other sections evaluate the degree to which the seasonal changes are experienced as

a problem and in which month of the year the subjects feel best or worst. These three scales allow classifying whether the subjects have:

1. Seasonal Affective Disorder Syndrome (SAD): GSS more than 10 and seasonal changes experienced as a moderate problem.
2. Seasonal affective disorder subsyndrome (S-SAD): GSS between 9 and 10 and seasonal changes experienced as a moderate problem or GSS more than 11 and seasonal changes experience as a mild problem.
3. No Seasonal affective disorder

The differences in allele and genotype frequencies between groups were assessed using χ^2 analysis or Fisher's exact test ($p<0.05$). ANCOVA was used to test for associations between MEQ and SPAQ scores and genotypes, with age and gender as covariates. Bivariate correlation was performed between MEQ scores and age (Pearson test). A p value <0.05 was considered as significant.

RESULTS

1. PROJECT 1

MELANOPSIN RETINAL GANGLION CELLS IN HEREDITARY OPTIC NEUROPATHIES

1.1. Melanopsin RGCs in DOA and age-matched control

To expand our previous observations of mRGCs sparing in LHON patients we studied a further 87-year-old patient with DOA, who belonged 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 subsequently 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 (**Figure 20**).

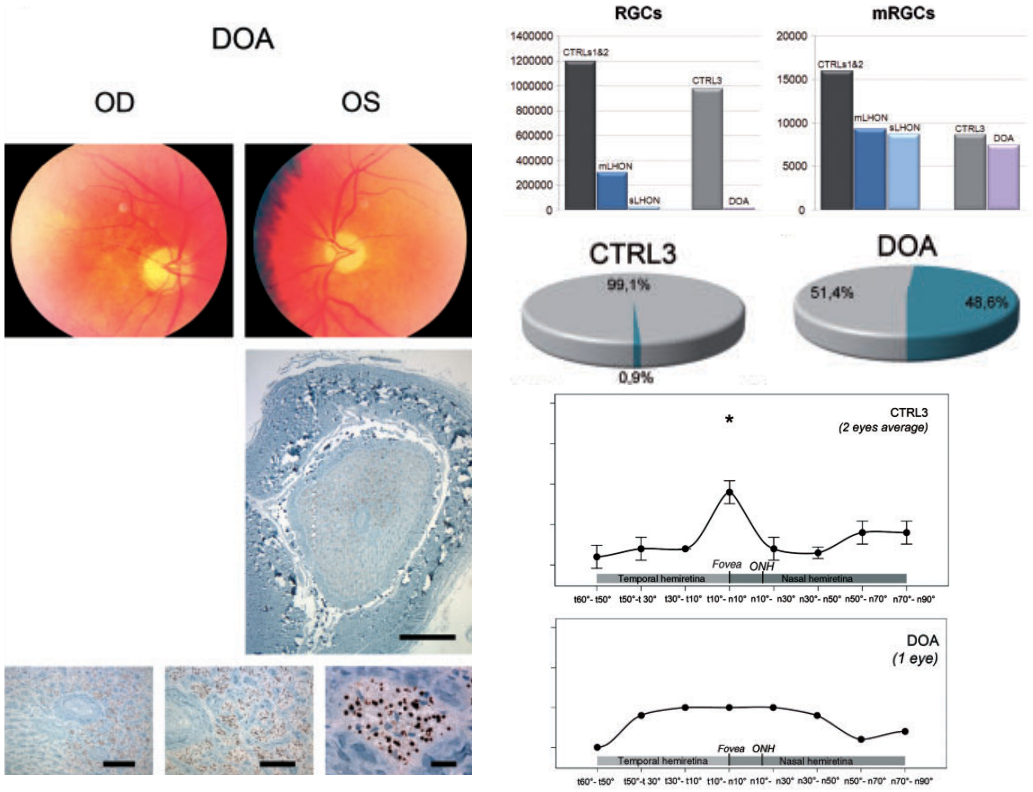


Figure 20. Left panel: OD and OS fundus oculi of DOA patient; OS optic nerve cross-section at low an higher mag. Right panel: number of RGCs, mRGCs, mRGCs/RGCs ratio and retinal distribution of mRGCs in DOA patient

The histopathology of this subject's eyes has been previously reported (Kjer et al., 1983). We had available the left eye and optic nerve for this study (thanks to courtesy of Dr. Paul Kjer). The axonal count in the optic nerve cross-section, performed in the same fashion as for the study of LHON cases, showed a dramatic decrease to 15000 axons as compared with the 977851 axons counted in the 85-year-old age-matched control eye (94% loss). Manual counting of mRGCs in the retinal specimens immunostained for melanopsin showed a density of 7 cells/mm^2 for the DOA subject and 8.4 cells/mm^2 for the control subject. Thus, the percentage of mRGCs, compared with the total number of RGCs, was 48.6% for the DOA subject and 0.9% for the age-matched control. Melanopsin RGCs were non-uniformly distributed in the 85-year-old control, with a significant enrichment in the parafoveal region, whereas the retina from the DOA case showed a flat distribution (**Figure 20**). These confirmatory results, by studying a single patient with a different mitochondrial hereditary optic neuropathy such as DOA, were added to those previously obtained with LHON patients and have been published in the journal Brain in 2010 (La Morgia et al., 2010) (see Annex 1).

1.2. Pupillometric evaluation of LHON patients and controls

We evaluated by pupillometry (see methods) 13 LHON patients and 16 age-matched controls. All LHON patients showed a pupil response both with blue and red light showing variable degrees of preservation compared to controls. As a group, LHON patients had significantly lower peak amplitude of the pupil response both with the blue ($p=0.004$) and red light ($p=0.007$) and a significantly less sustained response to both blue ($p=0.001$) and red light ($p<0.001$) compared to controls. However, at single subject level, considering as pathological the values below 2SD from the mean of controls, only one patient had all four parameters pathologic, four patients had three, one patient had two, and two patients had one (**Figure 21).**

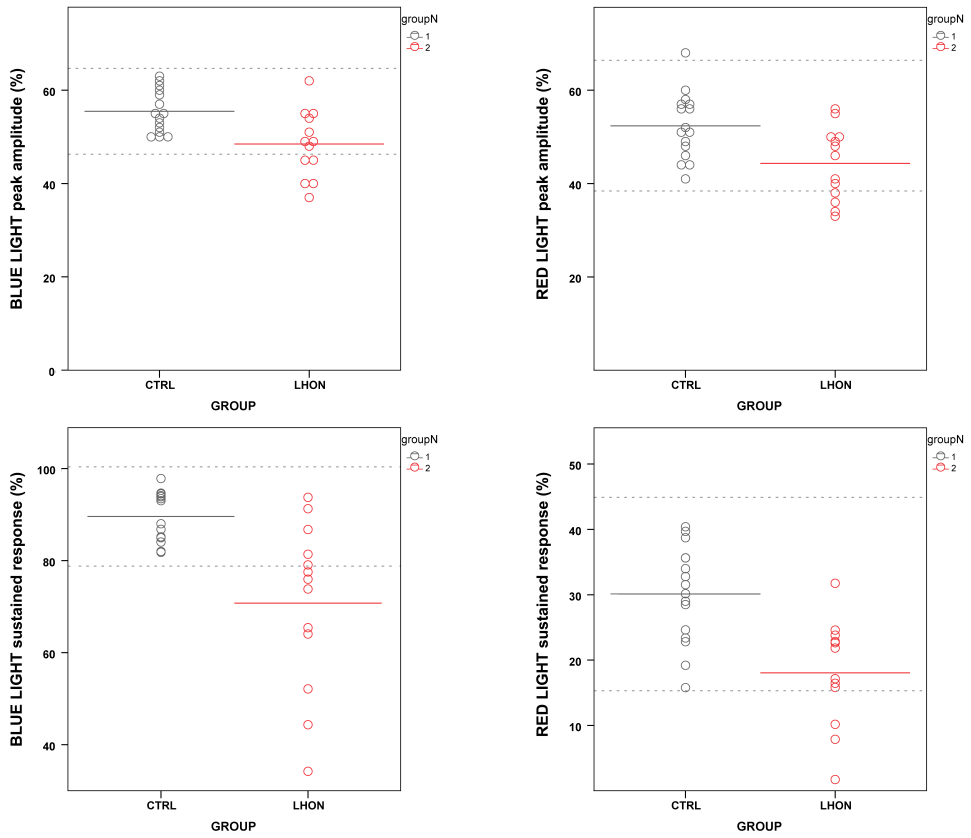


Figure 21. Pupillometry results for controls and LHON

Examples of patients with pupil response comparable to controls and patients with a weaker pupil response both at blue and red light are provided in **Figure 22**.

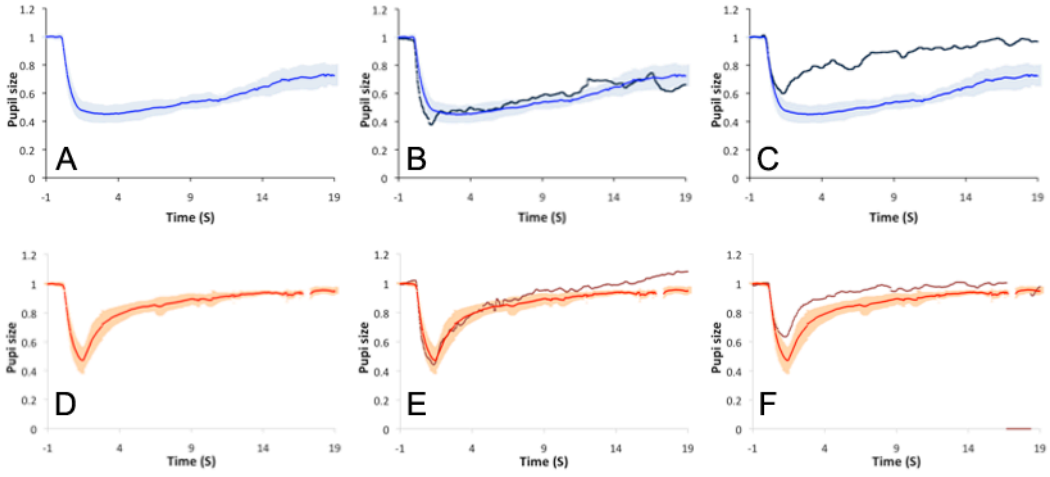


Figure 22. (A-C): examples of sustained response at blue light for controls, LHON patient with a clear melanopsin response and LHON patient with a weaker pupil response. (D-F): examples of sustained response at red light for controls, LHON patient with a clear melanopsin response and LHON patient with a weaker pupil response

The amplitude of the peak response significantly correlated with the sustained response with blue light (p=0.001) in LHON patients (**Figure 23**).

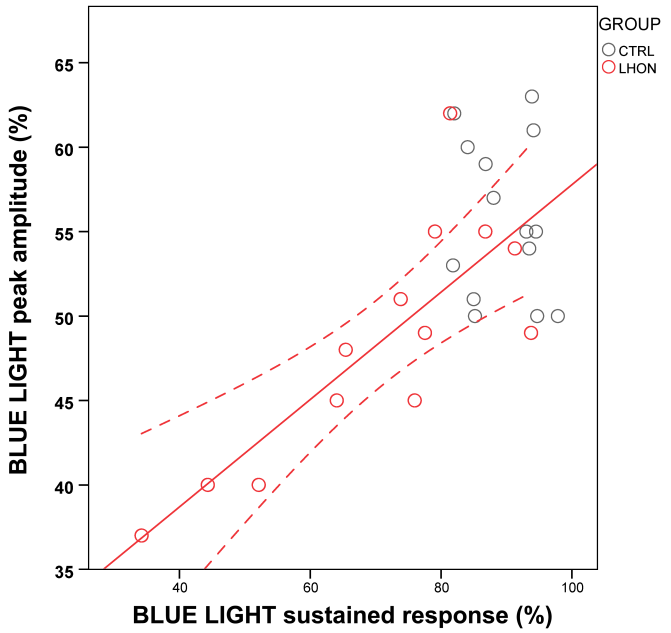


Figure 23. Correlation between peak amplitude and sustained response at blue light in LHON and controls

The pupil light response did not correlate with clinical parameters such as visual acuity and disease duration and the number of abnormal pupil parameters did not correlate with visual acuity (**Figure 24**).

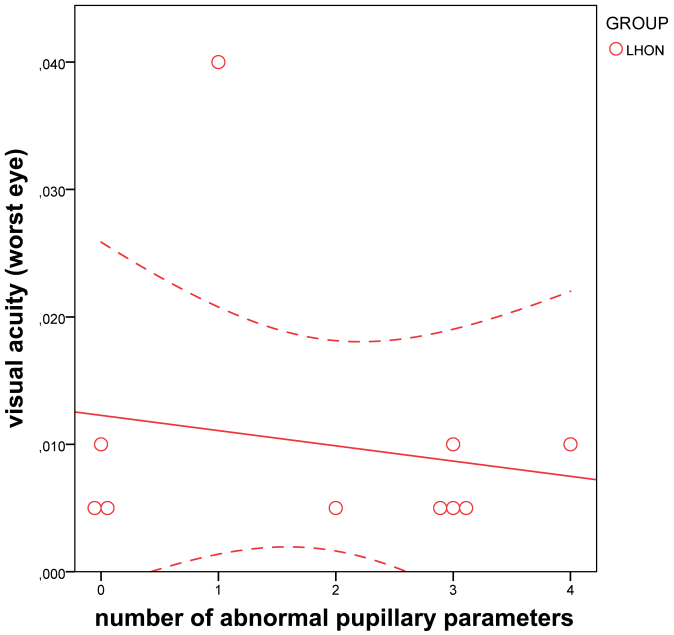


Figure 24. Correlation between the visual acuity of the worst eye and number of abnormal pupillary parameters

1.3. Cell studies.

The conclusions of our study on mRGCs relative sparing in mitochondrial hereditary optic neuropathies pointed to the question of why and how the mRGCs are metabolically resistant to mitochondrial dysfunction. To further investigate this issue we hypothesized that expression of the photopigment melanopsin, specific of mRGCs, may be responsible for their metabolic robustness.

We used the HEK cells, engineerized to express melanopsin under a promoter controlled by tetracycline (provided by Dr Hannibal and Birgitte Georg, Dept. Clinical Biochemistry, Bispebjerg Hospital, Copenaghen, Denmark), to study cell viability under different paradigms of metabolic stress and light exposure, as detailed in the methods section.

The effects of rotenone and TBH exposure at increasing concentrations of HEK cells without or with melanopsin expression and withour or with the addiction or retinal are shown in **Figure 25** A-B (darkness) and **Figure 25** C-D (30 minutes of blue light exposure). No significant differences in cell viability were observed in any of the conditions used. Furthermore, other experiments with the same paradigm, using white light and 24-hour light exposure, similarly failed to show any differences in cell viability (data not shown).

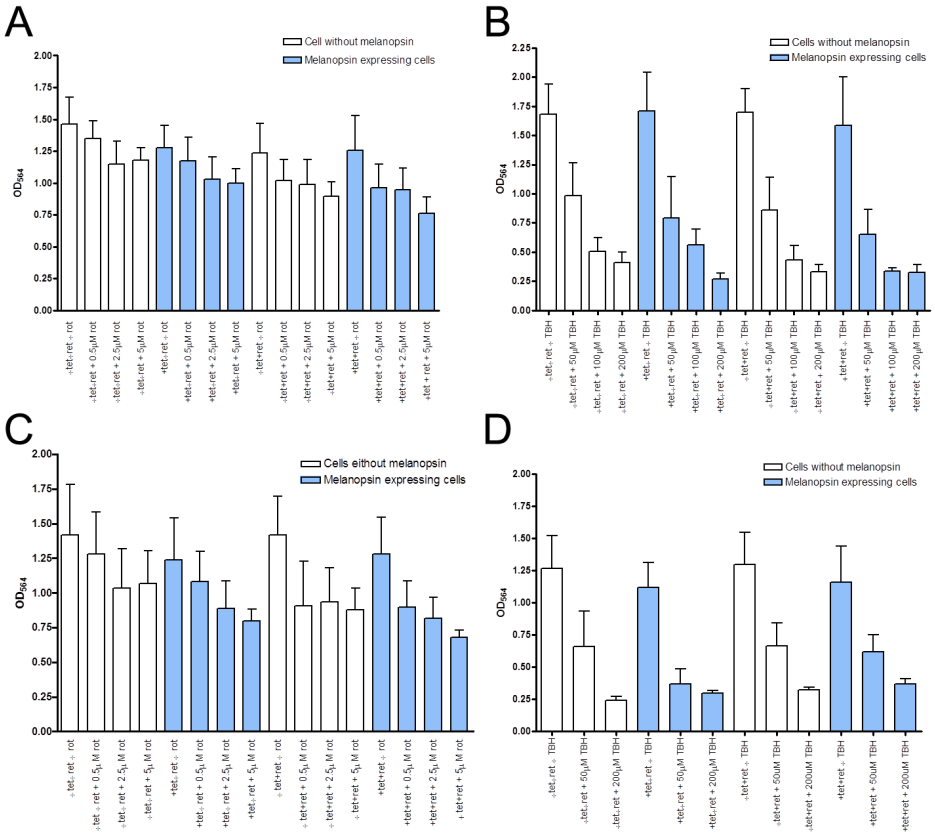


Figure 25. Results of cells studies in darkness (A-B) and after 30 min of blue light (C-D)

2. PROJECT 2

OPTICAL COHERENCE TOMOGRAPHY EVALUATION, ACTIGRAPHIC MONITORING AND SLEEP QUESTIONNAIRES IN NEURODEGENERATIVE DISORDERS

2.1. OCT evaluation

2.1.1. Alzheimer disease

We recruited 20 AD patients (40 eyes), of which two patients were excluded due to co-morbidity of glaucoma. Of the remaining 36 eyes enrolled, seven were excluded based on the previously established criteria (see methods) or because the OCT exam was technically not reliable. Three of the AD patients enrolled had non- specific visual complaints (difficulty in focusing objects). Overall, we included 18 AD patients (71.78±11.2; range 51-84; 10 males) and 74 age-matched controls (mean age 69.1±8.1; range 52-85; 31 males), for which one randomly chosen eye was used for statistical analysis. The average RNFL thickness was reduced in AD patients compared to controls (p=0.034) (**Table 8**, **Figure 26**). Considering separately the four quadrants, the RNFL thickness of superior (p=0.009) and nasal (p=0.016) quadrants was significantly thinner in AD patients compared to controls (**Table 8**). Demographic and the main clinical findings are summarized in **Table 8**.

	AD (N=18)	CONTROLS (N=74)	ANCOVA
GENDER	10M; 8F	31M; 43F	
AGE	71.78 ± 11.2 (51-84)	69.1 ± 8.1 (52-85)	
DIS DURATION	5.3 ± 2.6 (1-9)		
MMSEc	18.6 ± 3.4 (13.5-26.1)		
RNFLavg (microns)	87.8 ± 8.7 (71-104)	95.8 ± 12.3 (68-130)	*p=0.034
RNFL-T (microns)	67.9 ± 10.5 (49-84)	69.3 ± 10.5 (41-101)	p=0.942
RNFL-S (microns)	103.8 ± 13.6 (83-124)	117.1 ± 17.6 (81-152)	*p=0.009
RNFL-N (microns)	63.5 ± 11.7 (37-86)	74.3 ± 15.3 (48-133)	*p=0.016
RNFL-I (microns)	115.6 ± 16.5 (91-158)	122.4 ± 18.1 (85-154)	p=0.284

Table 8. OCT results in AD patients and controls

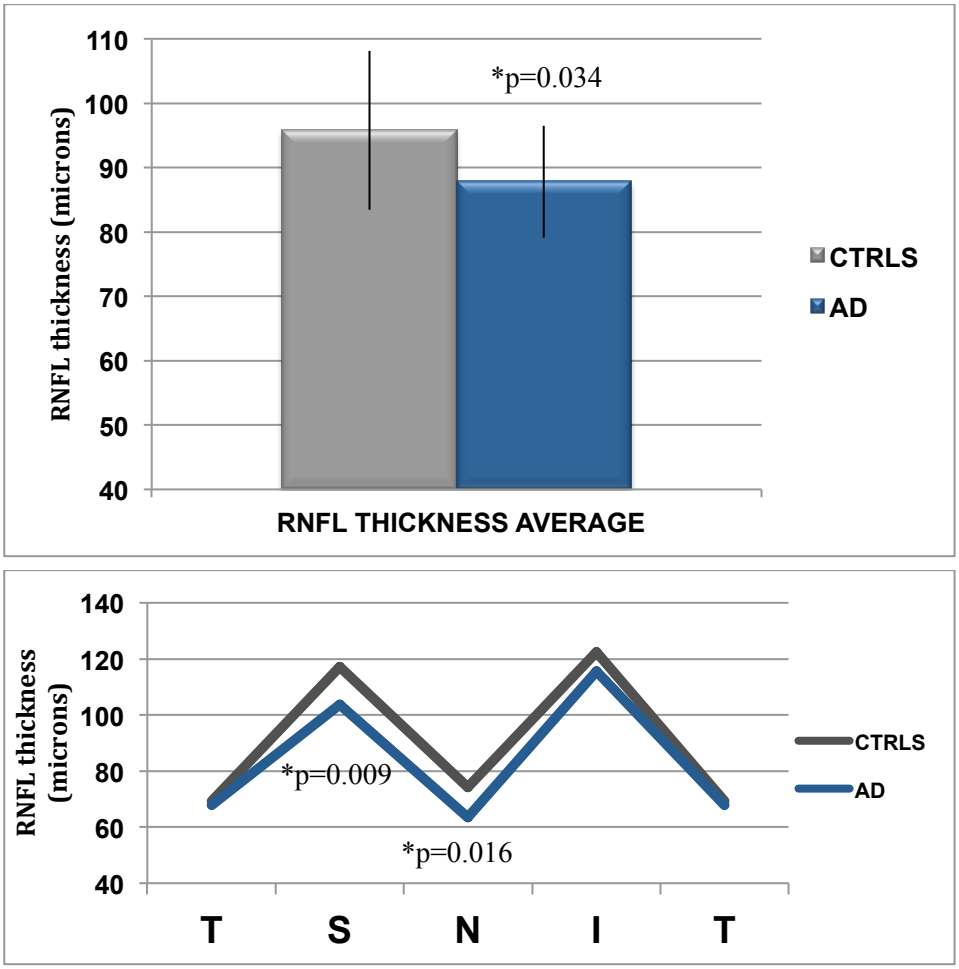


Figure 26. OCT results in AD patients. Average RNFL thickness (upper panel) and RNFL thickness of single quadrants (lower panel) in controls and AD patients

The Pearson test and regression analysis did not disclose any significant correlation between demographic or clinical parameters and RNFL measurements, except for age. Average RNFL thickness correlated with age in both controls (p=0.01; r=-0.289) and AD patients (p=0.005; r=-0.635) (**Figure 27**). Considering separately the four quadrants, the RNFL thickness of superior (p=0.05; r=-0.465) and nasal (p=0.04; r=-0.486) quadrants for AD patients, and of temporal (p=0.03; r=-0.247) and nasal (p=0.04; r=-0.235) quadrants for controls significantly correlated with age (data not shown).

Stratification of AD patients according to MMSEc score in mild and moderate AD showed that only for the moderate AD patients the average (p=0.05), superior (p=0.018) and nasal (p=0.006) RNFL thickness were significantly reduced compared to controls (data not shown).

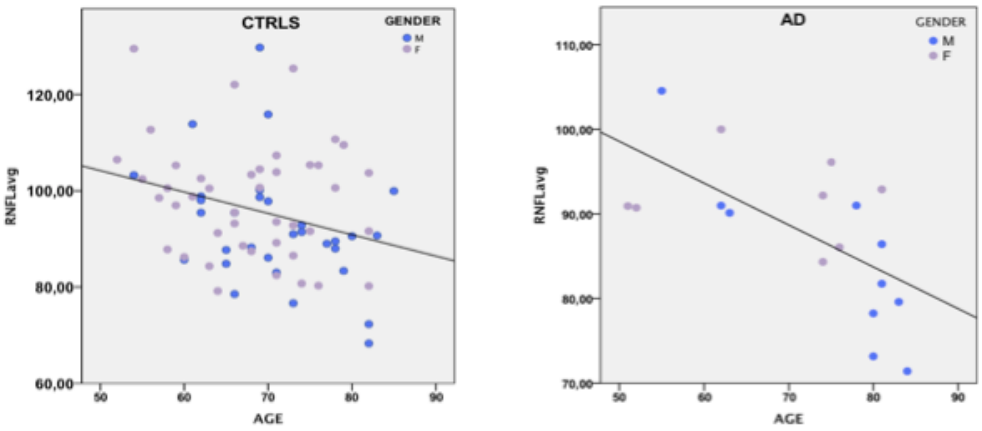


Figure 27. Correlation between RNFL average thickness and age for controls and AD patients

2.1.2. Mild Cognitive Impairment

We also evaluated by OCT 11 MCI individuals (73.09 ± 6.2; range 64-87; 6 males) and 54 age-matched controls (mean age 72.85 ± 5.6; range 64-85; 25 males). Clinical and demographic data are summarized in Table 9. None of the MCI patients had visual complaints. Three MCI eyes were excluded because the OCT exam was technically not reliable. Average RNFL thickness did not differ between MCI and controls (**Table 9**, **Figure 28**). Similarly, considering separately the four quadrants, there were no significant differences between MCI and controls (**Table 9**). As for AD patients, the Pearson test and regression analysis did not disclose any correlation between demographic or clinical parameters and RNFL measurements, except for age and only for the superior quadrant (p=0.004; r=-0.790) (data not shown).

Table 9.

	MCI (N=11)	CONTROLS (N=54)	ANCOVA
GENDER	6M; 5F	25M; 29F	
AGE	73.1 ± 6.2 (64-87)	72.8 ± 5.6 (64-85)	
DIS DURATION	3.9 ± 1.9 (2-8)		
MMSEc	24.9 ± 2.2 (20.3-27.4)		
RNFLavg (microns)	96.8 ± 10.4 (78-111)	94 ± 12.6 (68.3-129)	p=0.44
RNFL-T (microns)	68.1 ± 12.9 (46-97)	68.6 ± 12.7 (41-101)	p=0.972
RNFL-S (microns)	115.4 ± 19 (68-134)	115.4 ± 18.2 (81-152)	p=0.681
RNFL-N (microns)	81.3 ± 21.4 (57-134)	71.9 ± 13.3 (48-112)	p=0.054
RNFL-I (microns)	124.7 ± 14 (105-144)	120.3 ± 17.8 (85-176)	p=0.419

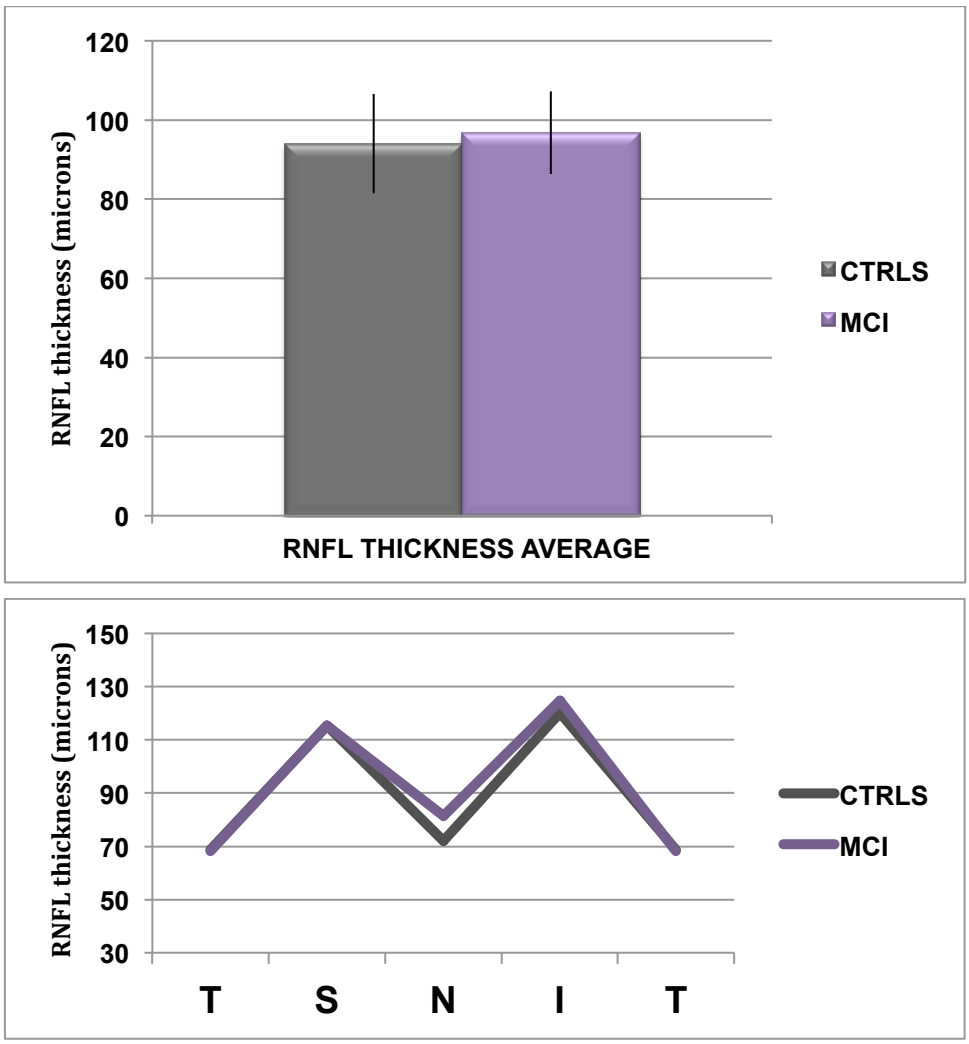


Figure 28. OCT results in MCI patients. Average RNFL thickness (upper panel) and RNFL thickness of single quadrants in controls and AD patients

2.1.3 Parkinson disease

We included 43 PD patients (mean age 65.6±8.4 years; range 46-83; 23 males) and 86 age-matched controls (mean age 65.5±10.7 years; range 46-85 years; 36 males). Among PD patients, three had a molecular diagnosis (two carried the G2019S *LRRK2* mutation and one a *PARK2* exon 2 deletion) and five had first-degree relatives affected with no molecular definition. We considered all these PD patients as genetic cases. For all patients we retrieved disease duration, UPDRS-III (off) score and stage of disease (Hoehn and Yahr). All PD patients included were on dopaminergic therapy. Disease duration was 9.1±6.5 years, Hoehn and Yahr stage of disease was 2.2±0.9 and UPDRS score was 25.9±12.4. Demographic, and the main clinical findings are summarized in Table 10. None of the PD patients had visual complaints. Five PD eyes (2 ipsilateral and 3 contralateral to the most affected body side) were excluded based on established criteria.

The average RNFL thickness was reduced in PD patients compared to controls, being close to significance (p=0.057) (**Table 10**, **Figure 29**). Considering separately the four quadrants, only the temporal RNFL thickness was significantly reduced in PD compared to controls (p=0.004) (**Table 10**). Post-hoc pairwise comparisons showed that the temporal RNFL thickness was significantly reduced for both the contralateral (p=0.008) and the ipsilateral (p=0.04) eye in PD compared to controls (**Figure 29**). The significance of RNFL thickness reduction in the temporal quadrant was maintained for the sporadic cases (p=0.006) and genetic cases (p=0.039) considered separately. Pearson test and multiple regression analysis did not disclose any correlation between genetic, demographic or clinical parameters and RNFL measurements, except for age. Average RNFL thickness correlated with age in both controls (p=0.001; r=-0.341) and PD patients (p=0.007; r=-0.412 and p=0.019; r=-0.371 for the eye ipsilateral and contralateral to the most affected body side respectively) (data not shown).

The results of OCT evaluation in PD are currently in press in the European Journal of Neurology (La Morgia et al., 2012b) (see Annex 3).

	PD IPSI EYE (N=41)	PD CONTRA EYE (N=40)	CONTROLS (N=86)	ANCOVA
GENDER	24 M; 19F		34M; 52F	
AGE	65.6 ± 8.4 (46-83)		65.5 ± 10.74	
DISEASE DURATION	9.1 ± 6.5 (3-28)			
UPDRS (OFF)	25.9 ± 12.4 (10-61)			
HY stage (OFF)	2.21 ± 0.9 (1-5)			
RNFLavg (microns)	92.2 ± 12.8 (68-121)	91.9 ± 12.1 (71-121)	97 ± 12.4 (68-130)	p=0.057
RNFL-T (microns)	62.3 ± 10.4 (45-86)	60.9 ± 12.8 (33-87)	68.5 ± 12.3 (41-101)	p=0.004*
RNFL-S (microns)	115.2 ± 17.4 (83-150)	112.4 ± 15.6 (76-144)	118.9 ± 17.4 (81-152)	p=0.179
RNFL-N (microns)	72.2 ± 18.3 (39-111)	72.9 ± 17.7 (52-114)	76.5 ± 15.7 (48-133)	p=0.362
RNFL-I (microns)	119.3 ± 19.9 (75-167)	121.5 ± 18.6 (79-161)	124.1 ± 18.6 (85-184)	p=0.426

Table 10. OCT results in PD and controls. Post-hoc pairwise comparisons: controls vs PD ipsilateral eye (p=0.04); controls vs PD contralateral eye (p=0.008)

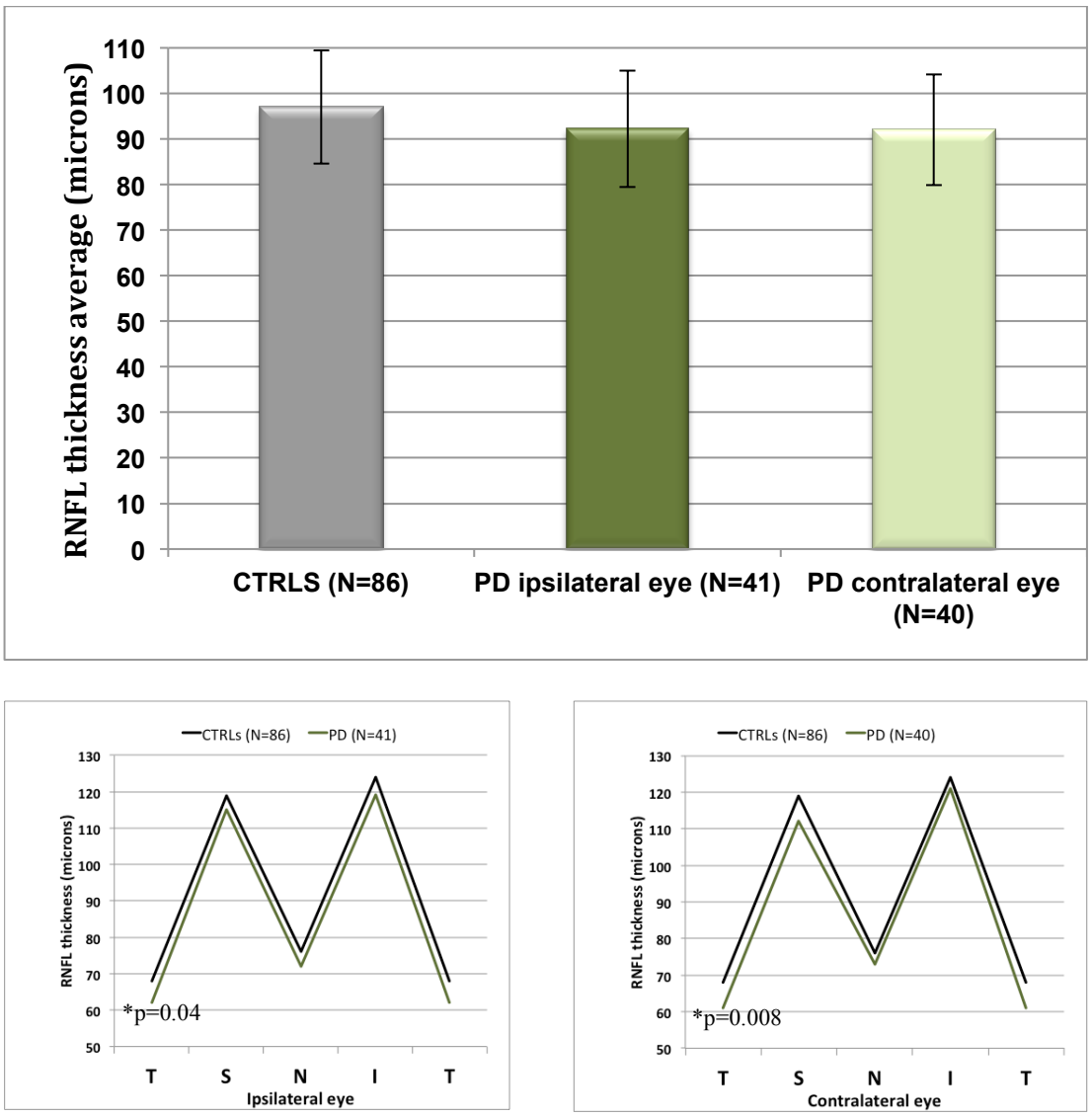


Figure 29. RNFL thickness results. Asterisks indicate statistical significance after post-hoc pairwise comparisons. A. The average RNFL thickness is shown. B. The temporal RNFL thickness is shown C. The RNFL thickness of temporal, superior, nasal and inferior quadrants is shown for control eyes (black line) and for ipsilateral PD eyes (gray line). D. RNFL thickness of temporal, superior, nasal and inferior quadrants is shown for control eyes (black line) and for contralateral PD eyes (gray line)

2.2. Actigraphic evaluation in AD, MCI and PD

Actigraphic monitoring of rest-activity rhythm for 7 days was performed in 15 AD patients, 7 MCI, 9 PD patients, all of them being subgroups of the cohorts investigated by OCT (see section 2.1), and 10 controls. "Bad" hours represent 0.5% of the total recording time (in particular 12 hours for AD, 1 hour for MCI, 7 hours for PD and 4 hours for controls).

Clinical and demographic data of patients and controls are summarized in **Table 11**.

All but 4 AD patients were on therapy for dementia (4 on donepezil, 2 on rivastigmine, 3 on memantine, 2 on galantamine and one on both galantamine and rivastigmine). MCI patients had multiple-domain (n=2), single-domain attentional (n=2) and single-domain amnesic cognitive dysfunction (n=3). All PD patients were on dopaminergic therapy (**Table 11**).

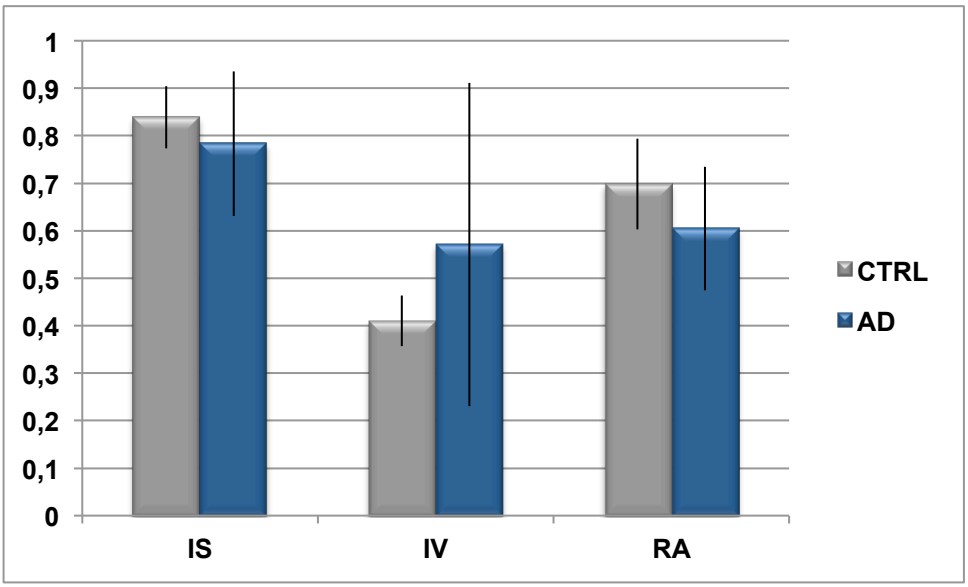
		MIN	MAX	MEAN	SD
AD (n=15)	DISDUR	1.00	9.00	4.9	2.5
	MMSEc	13.4	26.1	18.9	3.9
	AGE	52	84	70.9	10.3
MCI (n=7)	DISDUR	2.00	8	4	2.2
	MMSEc	20.3	27.5	24.8	2.6
	AGE	64	78	72.4	5.1
PD (n=9)	DISDUR	3	18	9.4	4.4
	UPDRS_OFF	13.3	43.9	24.1	11.5
	HY	1	3	1.8	0.8
	LEDD	200	580	400.6	135.8
	AGE	50	76	64.3	8.2
CTRLS (n=10)	AGE	54	80	65.8	7.5

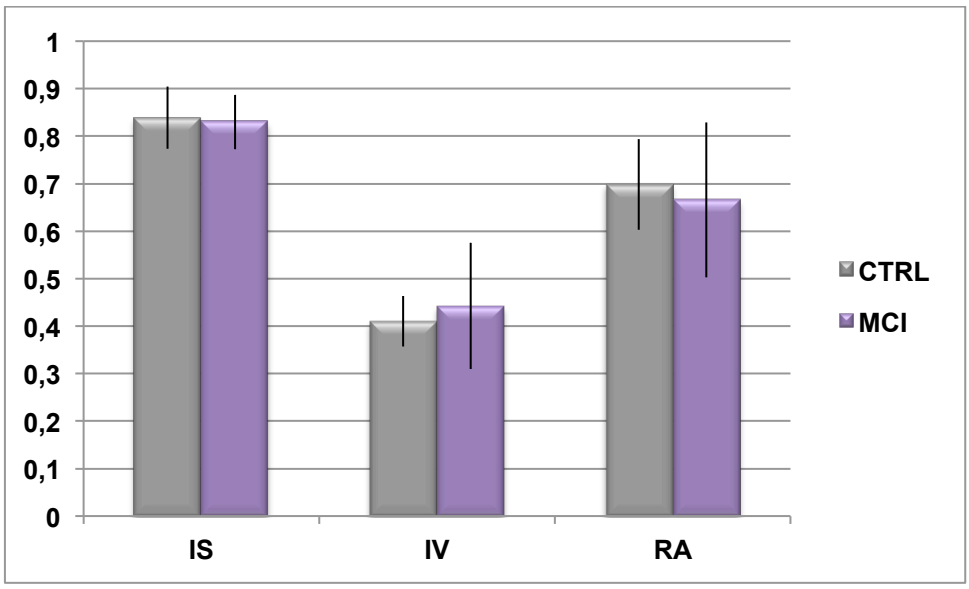
Table 11. Clinical and demographic data of patients and controls included in the actigraphic study

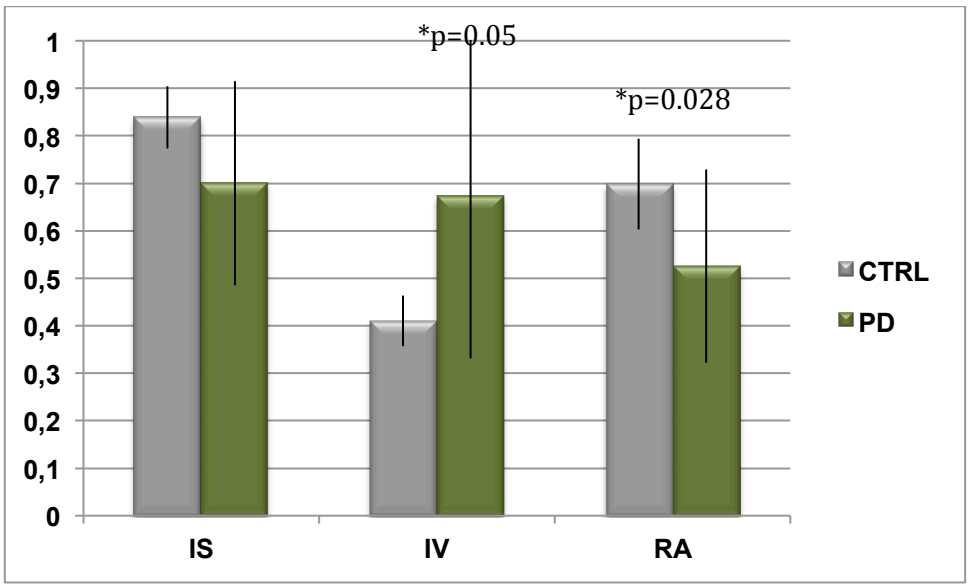
Analysis of actigraphic data using non-parametric methods (see methods) demonstrated a tendency towards an increased intradaily variability (IV) (p=0.098) and reduced relative amplitude (RA) (p=0.057) for AD patients compared to controls. AD patients also tended to be less active during the wake period (p=0.058). PD patients demonstrated a tendency towards a reduced interdaily stability (p=0.095) and showed an increased IV (p=0.05) and reduced RA (p=0.028) compared to controls. Moreover, PD patients were more active during the night period than controls (p=0.04). The same analysis applied to MCI individuals failed to reveal any differences from controls. Interdaily stability (IS) of rest-activity rhythm did not significantly differ between patients of all groups and controls (**Table 12**; **Figures 30-32**).

	AD (n=15)	MCI (n=7)	PD (n=9)	Ctrls (n=10)
IS	0.77 ± 0.15	0.83 ± 0.06	0.7 ± 0.21	0.84 ± 0.06
IV	0.57 ± 0.35	0.44 ± 0.13	0.67 ± 0.34	0.41 ± 0.05
RA	0.6 ± 0.13	0.67 ± 0.16	0.53 ± 0.2	0.7 ± 0.09
M10	261.9 ± 35.23	272.08 ± 15.4	273.7 ± 22.59	282.59 ± 15.2
L5	67.2 ± 28.49	57.5 ± 35.5	90.26 ± 47.96	50.5 ± 17.17

Table 12. Summary of actigraphic findings in AD, MCI, PD patients and controls



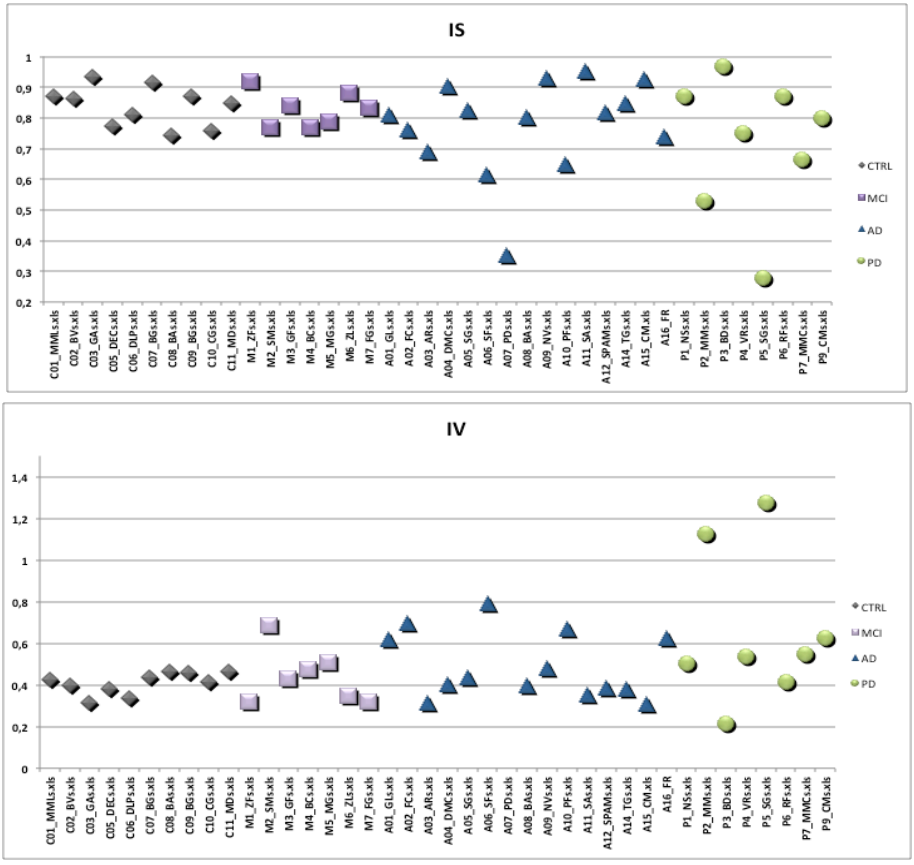




Figures 30-32. Circadian actigraphic parameters (IS, IV and RA) in AD, MCI and PD patients compared to controls

Individual data for IS, IV and RA results are shown in **Figure 33.**

Overall, the graphic representation of single individuals for these three parameters shows that both AD and PD patients present the highest variability, with scattered values, compared to controls. The MCI individuals had some degree of variability for the IV and RA parameters.



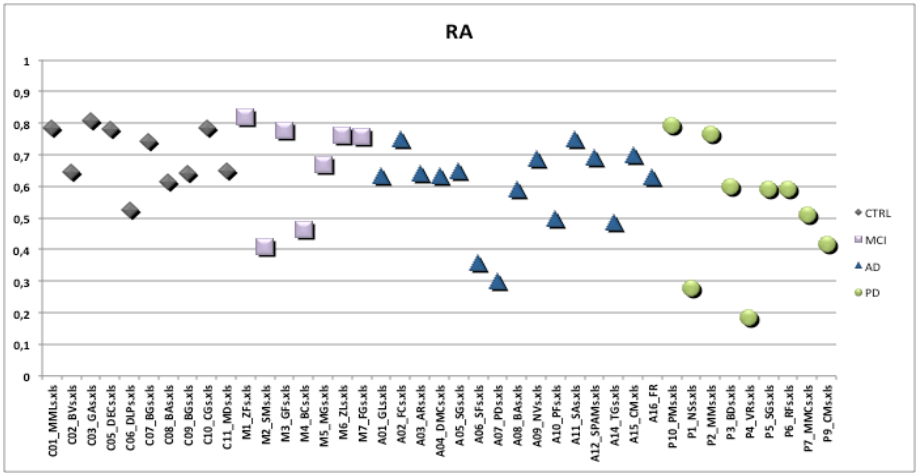


Figure 33. Individual circadian actigraphic results (IS, IV and RA) for controls, MCI, AD and PD patients

Zeta-transformed values [z=(v-μ)/σ] of actigraphic parameters, considering the mean and SD of the control group, are shown in **Figure 34.**

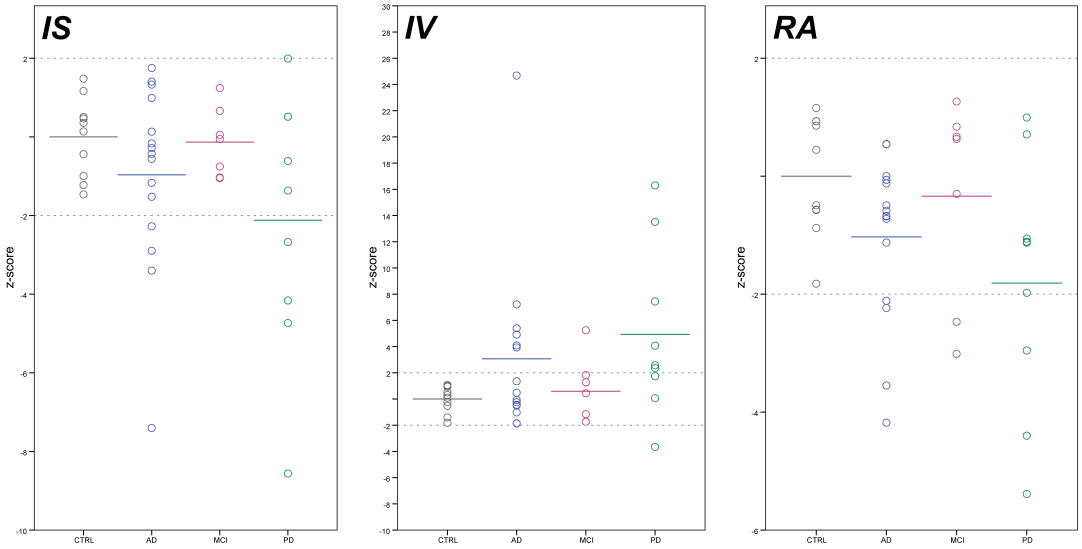


Figure 34. Z-scores for actigraphic parameters for controls and patients

None of the actigraphic data assessing rest-activity circadian rhythm significantly correlated with clinical parameters such as age, disease duration, MMSE score (for AD and MCI) and UPDRS-III score, H&Y stage of disease and LEDD (for PD). Furthermore, none of the actigraphic parameters assessing rest-activity circadian rhythm significantly correlated with OCT data. However, we found a tendency toward a correlation between RNFL average, RNFL superior and RNFL nasal with the actigraphic parameter IV in AD patients (**Figure 35**).

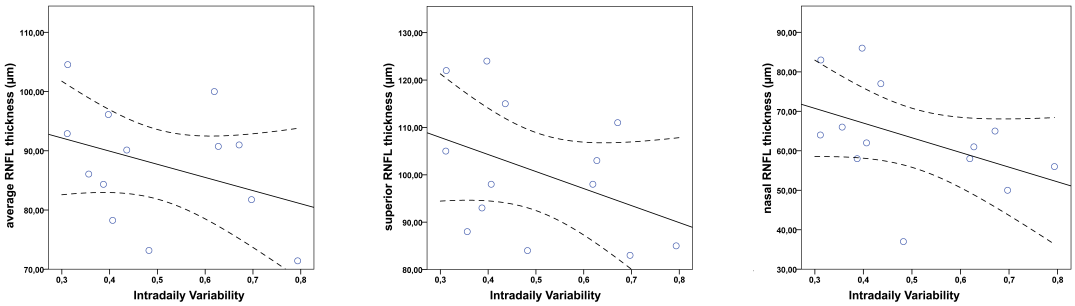


Figure 35. Correlation between intradaily variability and OCT measurements in AD patients

Stratifying AD patients by MMSEc in mild and moderate AD we did not find any significant differences between the two groups in terms of actigraphic parameters. However, considering the patients with at least one circadian parameter (IS, IV and RA) outside the 2SD from the mean of controls (n=6), for which OCT data were available, we found a significant correlation between IV, average (p=0.035), superior (p=0.045) and inferior (p=0.017) RNFL thickness (**Figure 36**).

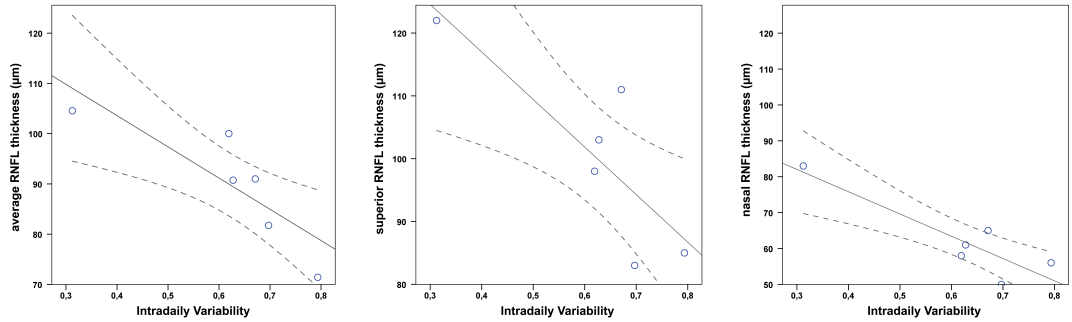


Figure 36. Correlation between IV and OCT parameters in "circadian-impaired" AD patients

An example of actigraphic recording for control, AD and PD patients is provided in **Figure 37.**

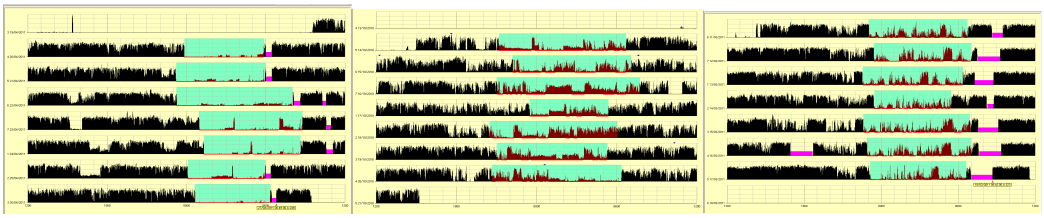


Figure 37. Examples of 7-days actigraphic recordings in control (left), AD (middle) and PD patients (right)

Examples of five-days profiles of hourly mean activity values are shown in **Fig. 38**.

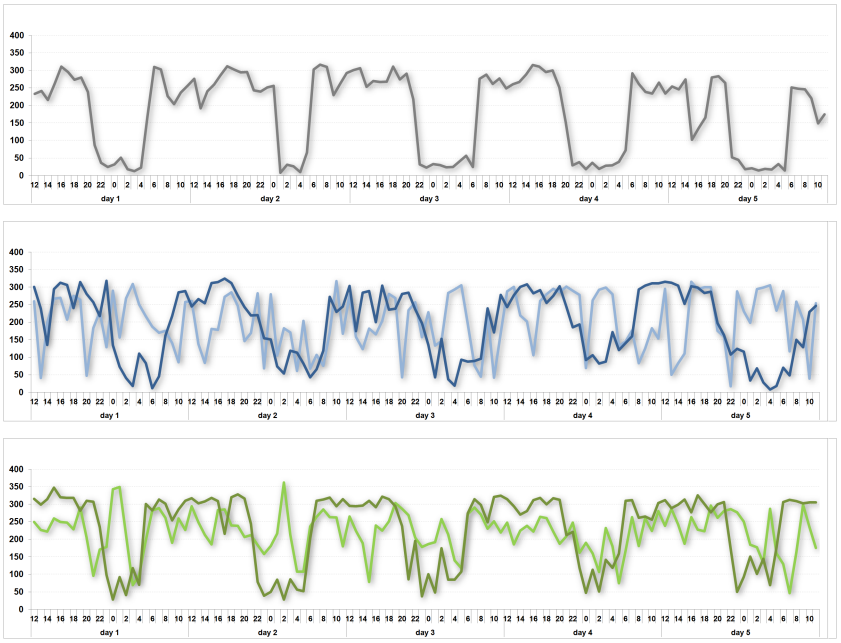


Figure 38. The upper panel shows the regular profile of a control subject; the middle panel shows the profiles of a circadian impaired AD patient (light blue) and of an AD patient with stable rhythm (dark blue); the lower panel shows the profiles of a circadian impaired PD patient (light green) and of a PD patient with stable rhythm (dark green).

Finally, the analysis of sleep parameters derived from actigraphic monitoring demonstrated a significant reduction of sleep efficiency in AD ($p<0.001$) and of both sleep minutes ($p=0.005$) and sleep efficiency ($p=0.001$) in PD patients compared to controls (**Figures 39-40**).

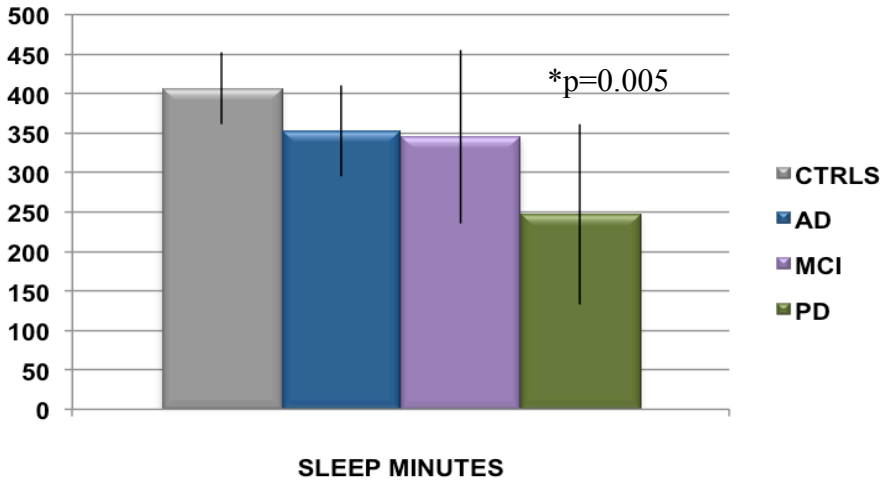


Figure 39. Sleep minutes (mean and SD) in controls, AD, MCI and PD patients

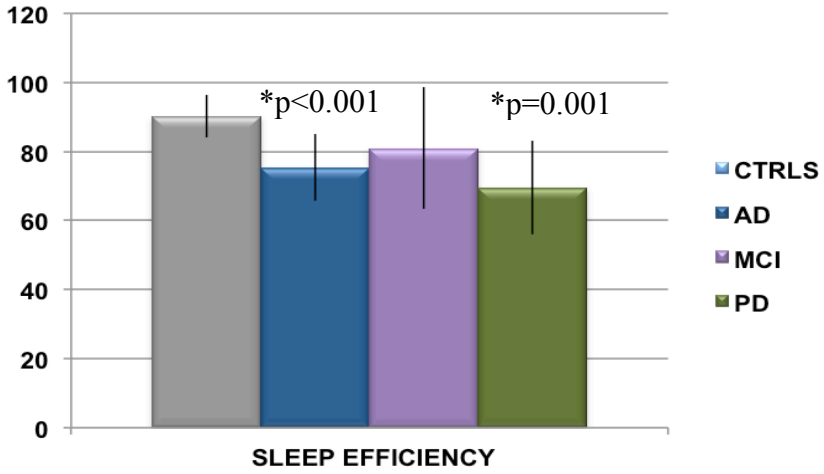


Figure 40. Sleep efficiency (mean and SD) in controls, AD, MCI and PD

Neither sleep minutes nor sleep efficiency significantly correlated with clinical parameters such as age, disease duration, MMSE score (for AD and MCI) and UPDRS-III score and H&Y stage of disease (for PD).

2.3. Sleep questionnaires.

We were able to collect sleep questionnaires for all AD patients, 5/7 MCI individuals, all PD patients and all controls. No significant differences emerged between patients and controls for subjective sleepiness evaluated by Epworth Sleepiness Scale (ESS), sleep quality evaluated by Pittsburgh Sleep Quality Index (PSQI) and risk of sleep apnea evaluated by Berlin test. In particular the risk of sleep apnea was high in 6/15 AD patients, 1/5 MCI patients, 3/9 PD patients and 2/10 controls. The results of the single items of the PDSS scale for PD patients are summarized in **Table 13**.

	Mean	SD
PDSS_1	6.5	2.1
PDSS_2	9	1.7
PDSS_3	8	2.4
PDSS_4	9.2	1.4
PDSS_5	7.8	2.2
PDSS_6	8.6	2.4
PDSS_7	9.7	0.6
PDSS_8	5.4	4.7
PDSS_9	9.9	0.1
PDSS_10	6.5	2.1
PDSS_11	9	1.7
PDSS_12	8	2.4
PDSS_13	9	1.4
PDSS_14	7.8	2.2
PDSS_15	8.6	2.4

2.4. Immunohistochemical analysis of AD post-mortem retinas and optic nerve cross-sections

We investigated sagittal retinal sections stained with melanopsin antibodies (Hannibal et al., 2004a) to recognize and count mRGCs (**Figure 41**) from 14 AD patients and 11 controls, adopting the same protocol used for the previous studies on mitochondrial optic neuropathies (La Morgia et al., 2010).
Examples of mRGCs located in the retinal ganglion layer (RGL) and inner nuclear layer (INL) and regular RGC are provided in **Figure 41**.

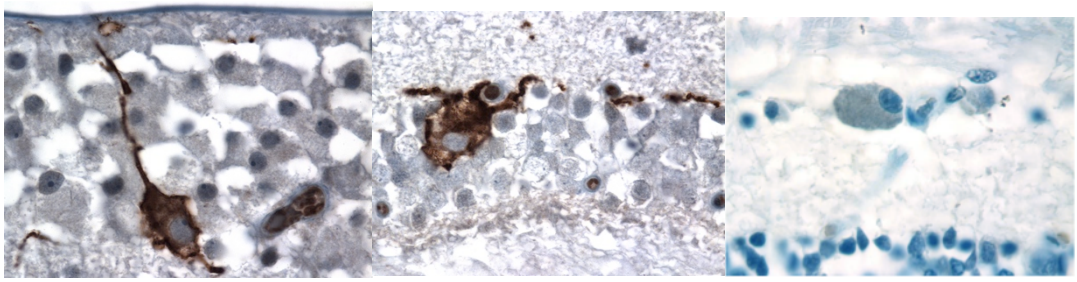


Figure 41. Examples of mRGC located in the RGL (left) and INL (middle), and regular RGC (right) (control3, 60 years)

We clearly distinguished by intensity of the melanopsin stain two types of mRGCs, intense (**Figure 41**) and light-stained (**Figure 42**), corresponding to the M1 and M2 types respectively (Schimdt et al., 2011b).

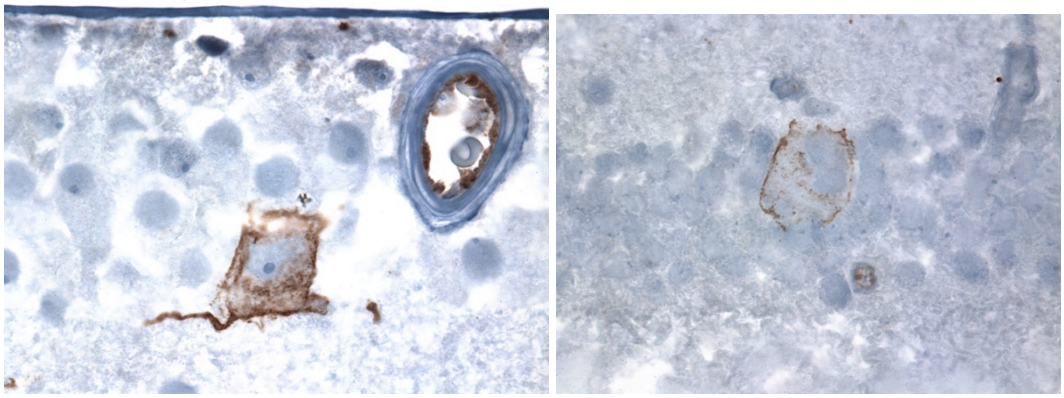


Figure 42. Examples of light-stained mRGCs (control 3, 60 ys)

Table 14 shows the main clinical and pathological features of the AD patients, as established by neuropathologic analysis of the brain, according to modified CERAD (Mirra et al., 1991) and NIA/Reagan criteria (Hyman et al., 1997).

ID	AGE	Pre-mortem dx	MMSE	CDR	DIS DUR (ys)	BRAAK STAGE
#778	62	Dementia	26 (59 ys)	n.a.	4	V (LBV)
#777	86	Vascular dementia	n.a.	n.a.	3	V
#783	64	AD possible	3 (64 ys)	3 (64 ys)	6	VI
#795	80	FTD	Unable to complete	4	n.a.	V
#801	75	AD	21 (72 ys)	n.a.	7	V
#800	96	AD possible plus LBD	0 (95 ys)	4 (95ys)	10	V
#781	80	AD	7 (80 ys)	3 (80 ys)	7	V-VI
#837	70	AD probable	25 (69 ys)	1 (69 ys)	4	V
#806	98	Dementia	9 (97 ys)	3 (97 ys)	8	V
#768	83	AD probable	14 (78 ys)	n.a.	7	VI
#769	71	FTD	n.a.	n.a.	14	VI
#776	98	AD possible	n.a.	2 (96 ys)	n.a.	V
#799	95	AD	17 (93 ys)	3 (93 ys)	18	V
#805	95	AD	n.a.	3 (92 ys)	11	V

Table 14. Demographic, clinical (MMSE and clinical dementia rating scale-CDR) and neuropathological data of AD cases; n.a.= not available; LBV: Lewy Body variant

The qualitative evaluation at light microscopy of the melanopsin-stained AD retinal sections compared to controls showed a grossly preserved structure of the retinal layers, including the RGL. We observed mRGCs located in both RGL and INL, as previously reported (Hannibal et al., 2004a). The morphology of mRGCs on the gross histological examination resembled that of controls. Some examples of mRGCs in AD patients are provided in **Figure 43**.

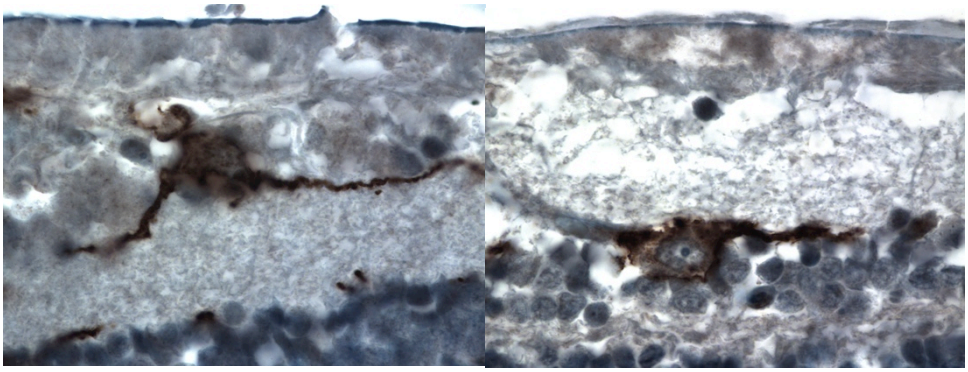


Figure 43. Examples of mRGCs in AD post-mortem retinas (#783)

However, in AD patients a frequent feature of fragmented dendrites was noted at high magnification (**Figure 44**), which was not evident at the same frequency in the age-matched controls.

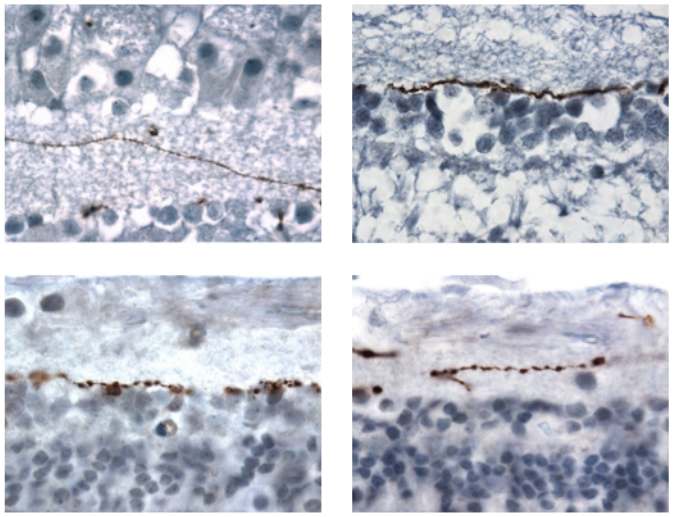


Figure 44. Examples for normal dendrites in controls (upper panels) (control 4, 74 ys and control 2, 85 ys) and fragmented dendrites in AD patients (lower panels) (#805, 95 ys)

Based on the gross histological examination, we recognized the presence of macular degeneration in one 98-year-old AD patient (#806) and of proliferative diabetic retinopathy in another 70-year-old AD case (#837). The retrospective evaluation of the clinical charts of these two patients confirmed the diagnosis of macular degeneration and diabetic retinopathy, respectively. Based on these comorbidities

involving retinal pathology we decided to exclude these two cases from the statistical analysis.

We also examined at light microscopy the optic nerve cross-sections from the same AD patients and controls for which we studied the sagittal retinal specimens for mRGCs counts. Optic nerves were embedded in epon plastic and stained with PPD to identify the myelin profiles, indicative of the single axons. At gross examination we identified one AD case (#777, 86 yrs male) with an extreme loss of axonal profiles compatible with non-arteritic ischemic optic neuropathy (NAION), which for this reason was excluded from the statistical analysis. All the remaining cases (n=11) showed an essentially preserved anatomy with some of the AD patients having a noticeable optic neuropathy with different degrees of axonal depletion (**Figure 45**), as previously reported (Hinton et al., 1986).

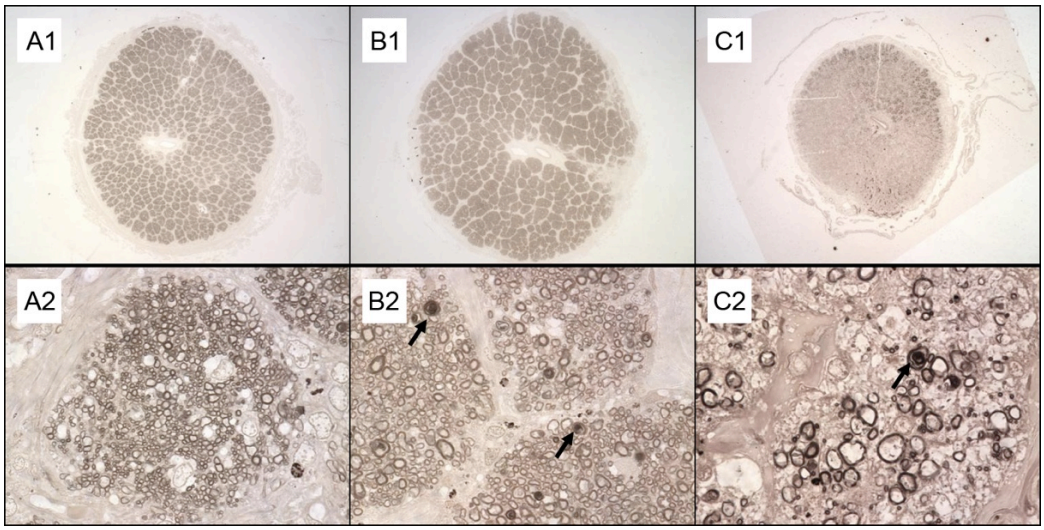


Figure 45. Comparison of human postmortem optic nerves in a control (A1), and AD patient (B1), and a patient with LHON (C1). Note higher density of axons in the control at higher magnification (A2) as compared to the AD (B2) and the LHON (C2) nerve tissues. Arrows in B2 and C2 point to degenerative profiles (magnification in A1, B1 and C1 is 25x; A2, B2 and C2 is 1000x).

Some examples of optic nerve cross-sections profiles from AD cases with different degrees of axonal loss are provided in **Figure 46** (#800, #799, #795).

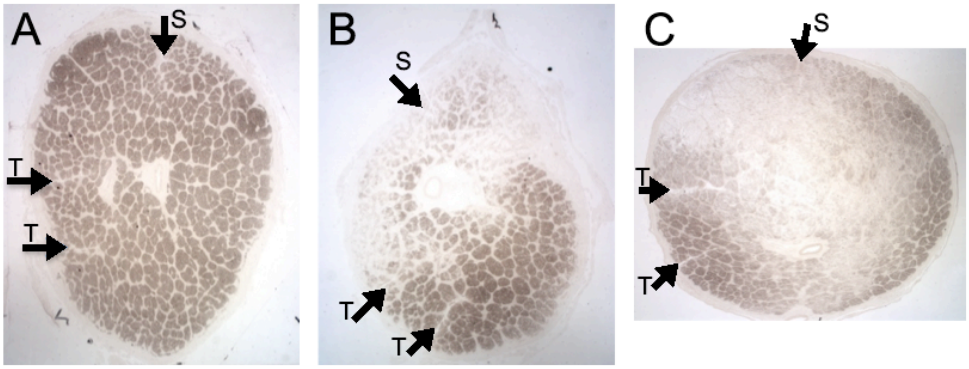


Figure 46. ON cross-sections of AD patients showing different degrees of axonal depletion

Interestingly, as shown by the above figures, the axonal loss affects the large fibers at the superior quadrant for the #799 and #795 AD, as well as nasal and inferior quadrants, whereas the temporal sector is mostly spared by neurodegeneration (**Figure 46**). Overall, after the excluded cases, the quantitative analysis and the following statistics have been performed on a set of 11 AD patients (81.7±13.1 years; 5 male and 6 females) and 11 age-matched controls (78.5±15.1 years; 7 males and 4 females).

Melanopsin RGCs were equally distributed in the RGL and INL in both AD and controls without differences between groups (**Figure 47**).

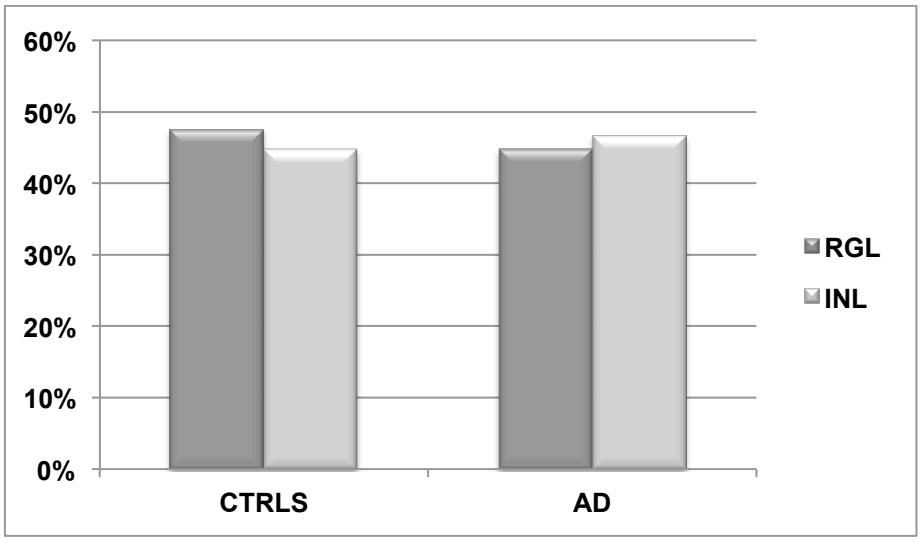


Figure 47. Melanopsin RGCs distribution in the RGL and INL for controls and AD

Melanopsin RGCs density was significantly reduced in AD patients compared to controls (p=0.008) (**Figure 48**).

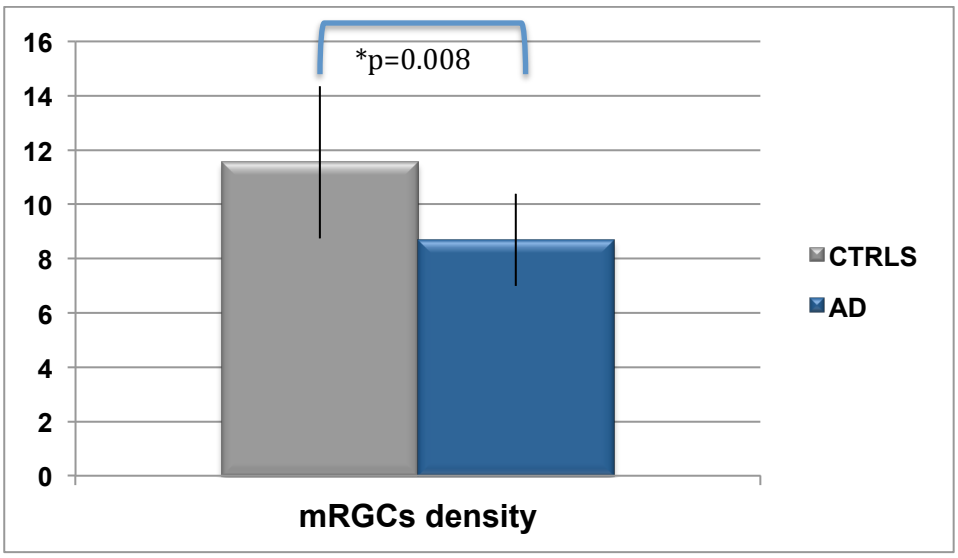


Figure 48. mRGCs density in controls and AD patients

Axonal count also showed a reduction in AD patients compared to controls, close to statistical significance (p=0.067) (**Figure 49**).

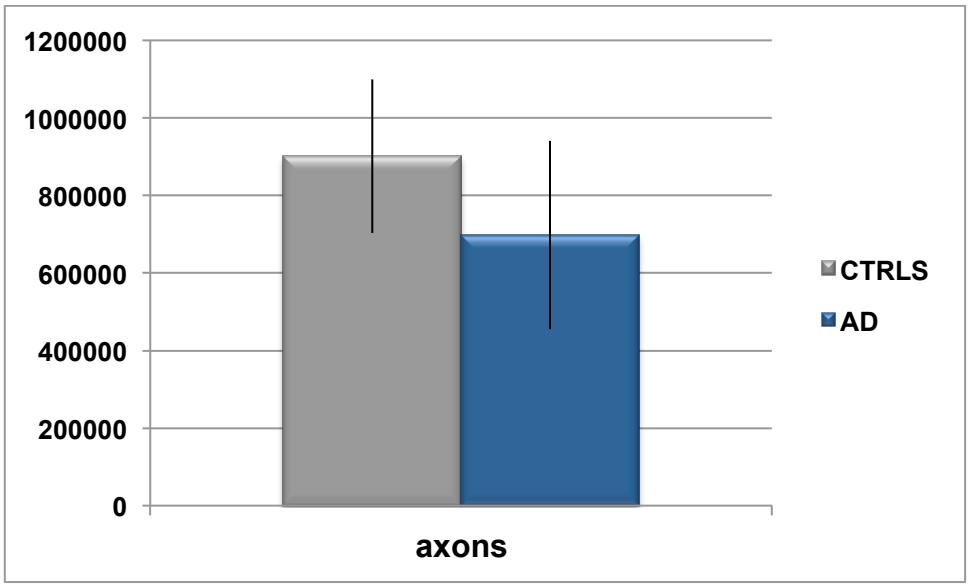


Figure 49. Axonal counts in controls and AD patients

As a result, the mRGCs/RGCs ratio, assuming that the axonal count equals the RGCs number, was comparable between AD patients and controls (p=0.683) (**Figure 50**).

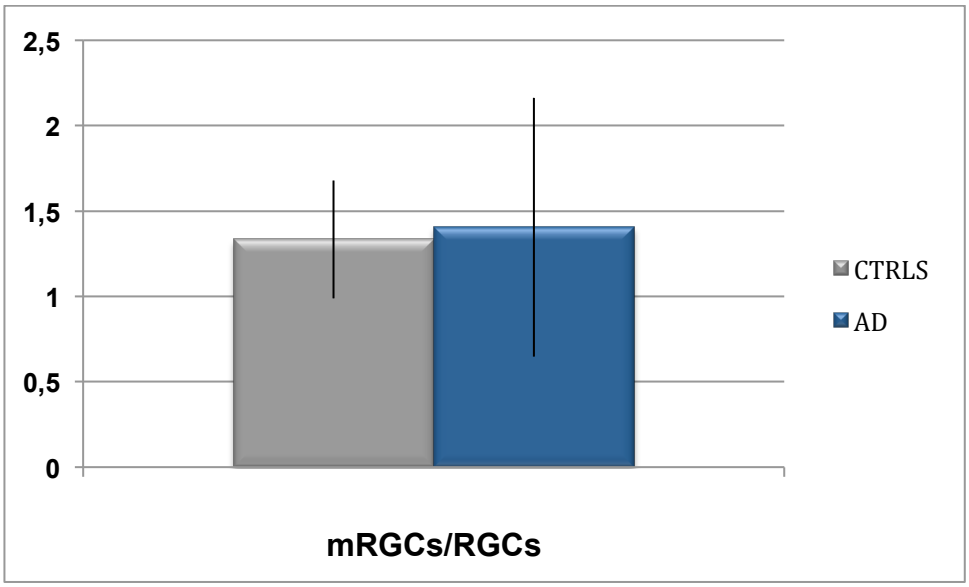


Figure 50. mRGCs/RGCs ratio in controls and AD patients

Both mRGCs density (p=0.033; r=-0.643) and axonal count (p=0.006; r=-0.763) significantly correlated with age only in controls (**Figure 51**).

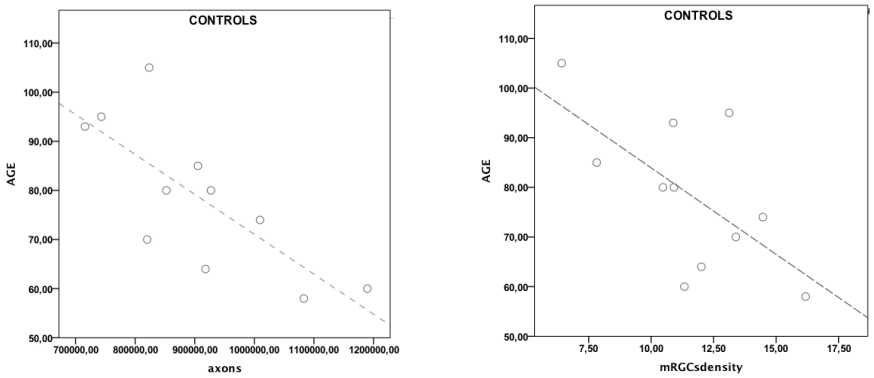


Figure 51. Correlation between axons, mRGCs density and age in controls

Stratifying all the previous data by decades we observed a decline of axonal count with age in both controls and AD. At difference, mRGCs density declined with age in controls, whereas in AD patients it was reduced across all ages. Remarkably, in the 60s and the 70s decades, this reduction of mRGCs density occurred in AD patients in the presence of an essentially normal number of axons in the optic nerve (**Figure 52**).

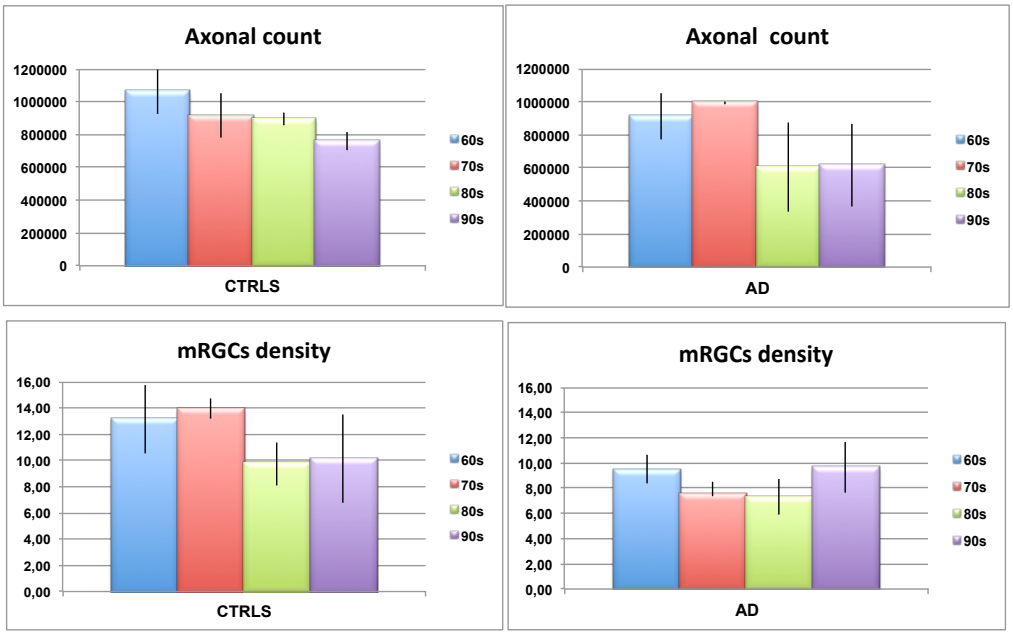


Figure 52. Axonal counts and mRGCs density by decades in controls and AD

Furthermore, we subgrouped controls and AD patients in four categories, using 80 years of age as a cut-off to distinguish “younger” and “older” individuals (controls <

80 ys, controls ≥ 80 ys, AD < 80 ys and AD ≥ 80 ys). Both mRGCs density (p=0.004) and axonal count (p=0.003) were significantly different between groups (**Figure 53, Table 15**).

				CI 95%	
		Standard error	Sig.	lower limit	upper limit
mRGCs density	youngCtrls vs youngAD	1.4	0.012	0.9089	9.1
	youngCtrls vs oldAD	1.2	0.005	1.2711	8.3
axons	youngCtrls vs oldAD	95894.4	0.004	108294.1	676513.7
	youngAD vs oldAD	102648.9	0.022	38078.7	646321.5

Table 15. Significant post-hoc comparisons (Bonferroni) are shown between groups

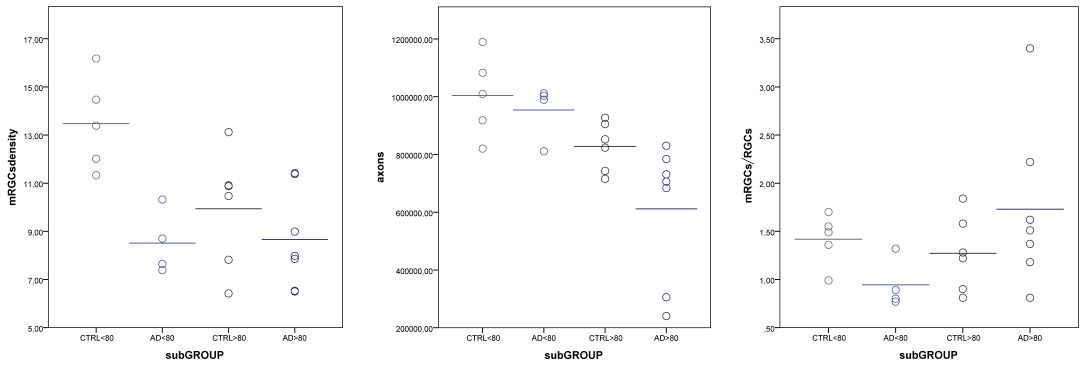


Figure 53. mRGCs density, axonal counts and mRGCs/RGCs ratio for subgroups

Considering separately males and females we did not observe any difference for the mRGCs density and axonal count in the control group, whereas a tendency towards a higher mRGCs density was evident for AD females compared to AD males (p=0.078) (**Figure 54**).

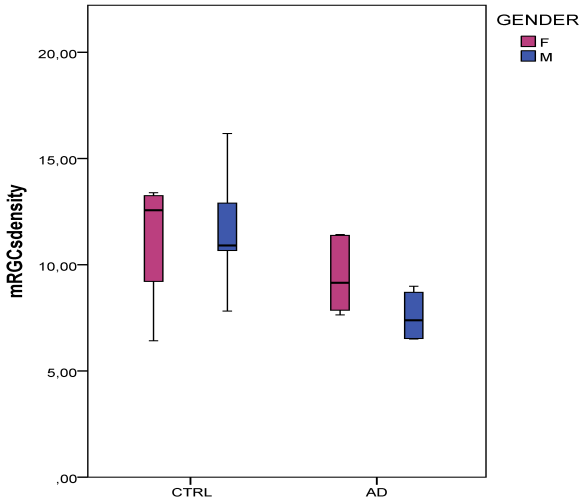


Figure 54. mRGCs density results by gender in controls and AD patients

Finally, we failed to observe any correlation between mRGCs density, axonal count and mRGCs/RGCs ratio neither with disease duration nor with Braak score in AD cases. However, a significant correlation was evident between mRGCs density and the B-score assessing the severity of neurofibrillary tangles (NFTs) deposition at the level of the superior/middle temporal cortex (p=0.014; r=-0.775), and the C-score assessing the severity of neuritic plaques (NPs) deposition at the lateral geniculate body of hippocampus (p=0.014; r=-0.710) (**Figure 55**).

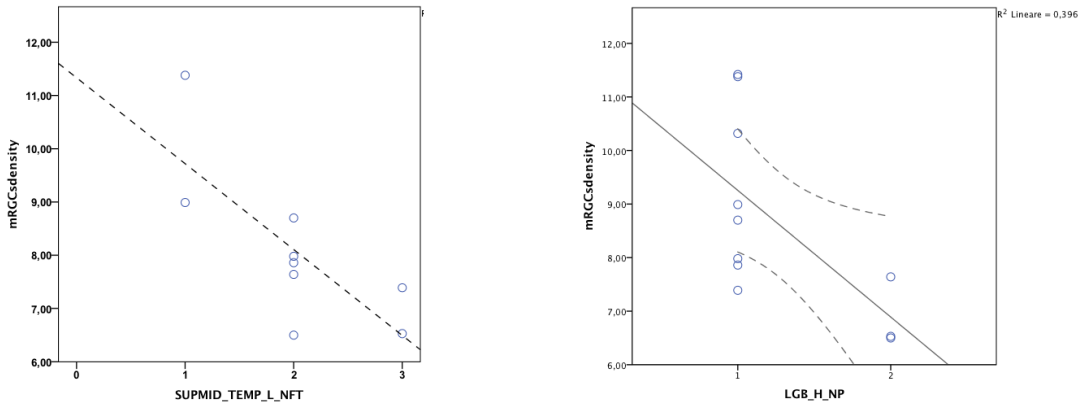


Figure 55. Correlation between the mRGCs density and NFTs-score in superior-middle temporal cortex and between the mRGCs density and NPs-score at lateral geniculate body of hippocampus

3. PROJECT 3

OPN4 GENE, CHRONOTYPES AND SEASONAL AFFECTIVE DISORDER

3.1. Genetic variant of the OPN4 gene and chronotypes

We assessed the genotypes (CC, TC and TT) related to the single nucleotide polymorphism (rs2675703-P10L-exon1) in the *OPN4* gene, previously associated with the seasonal affective disorder (Roecklein et al., 2009), in 314 subjects (mean age 31.8 ± 9.9 years; 103 male-32.8% and 211 females-67.2%) characterized for their chronotype by the Morningness Eveningness Questionnaire (MEQ).

The frequency of chronotypes is shown in **Table 16** and **Figure 56**.

Chronotype	FREQUENCY
EXTREME EVENING	20 (6.4%)
MODERATE EVENING	44 (14%)
INTERMEDIATE	190 (60.5%)
MODERATE MORNING	54 (17.2%)
EXTREME MORNING	6 (1.9%)

Table 16. Frequency of chronotypes

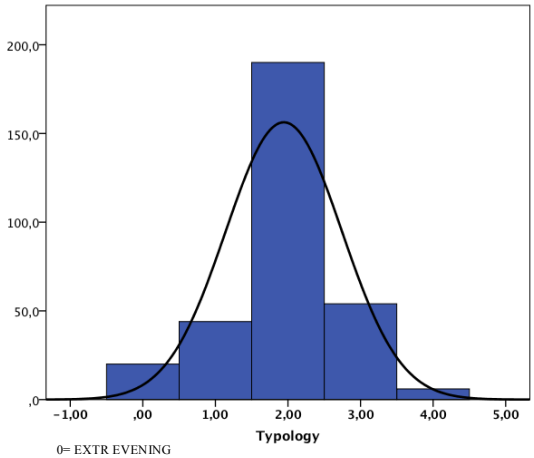


Figure 56. Chronotype distribution

The distribution of chronotypes was not different by gender (**Figure 57**).

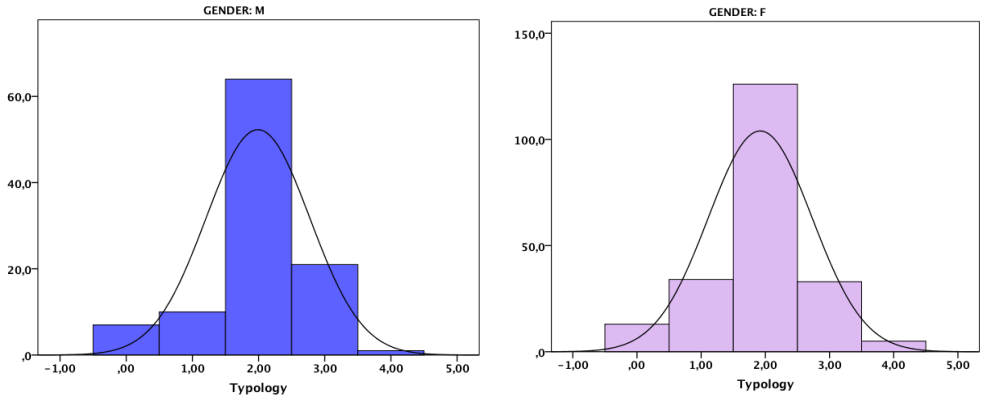


Figure 57. Distribution of chronotypes by gender

We found a significant correlation between MEQ score and age (p=0.007; r=-0.152) (**Figure 58**).

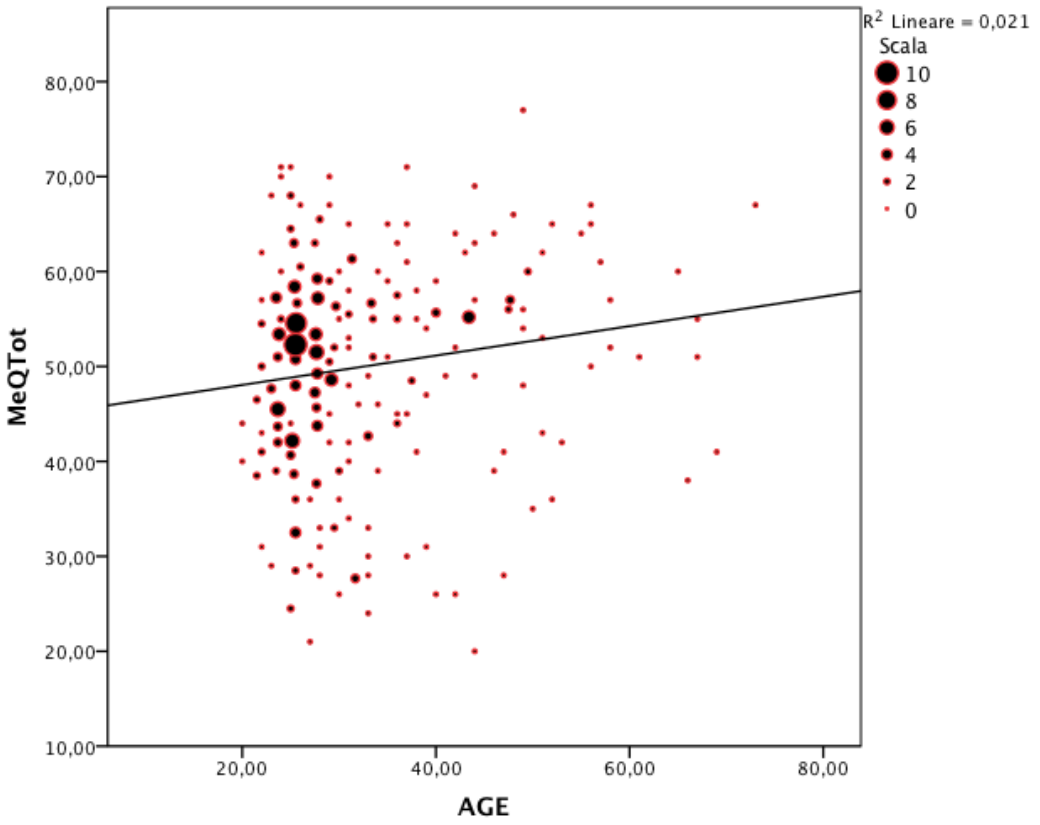


Figure 58. Correlation between MEQ score and age

Neither the genotypes (CC, TC and TT) nor the allelic distribution were significantly different between chronotypes, either considering the five categories separately (p=0.616) or grouped (p=0.428) (**Table 17, 18**). These results were not different if we included only subjects below 40 years of age (data not shown).

	EXTR EVENING	MOD EVENING	INT	MOD MORNING	EXTR MORNING	Tot
CC	12	27	130	38	2	209 (66.5%)
TC	7	15	51	15	3	91 (29%)
TT	1	2	9	1	1	14 (4.5%)
Tot	20	44	190	54	6	314

Table 17. Distribution of the five categories of chronotype by P10L genotype

	EVENING	INT	MORNING	Tot
CC	39	130	40	209 (66.6%)
TC	22	51	18	91 (28.9%)
TT	3	9	2	14 (4.5%)
Tot	64	190	60	314

Table 18. Distribution of the three categories of chronotype by P10L genotype

The distribution of the genotypes by chronotype and by MEQ score is shown in **Figure 59.**

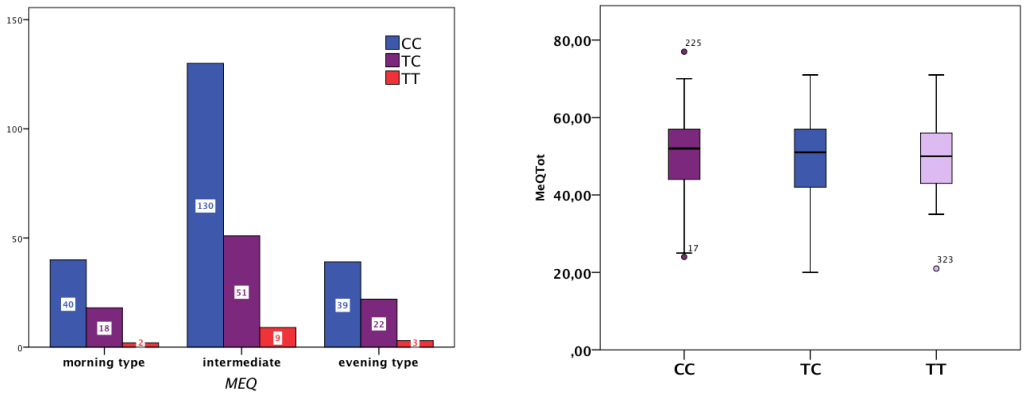


Figure 59. Distribution of P10L genotypes for the three chronotypes (left). MEQ score of the three P10L genotypes (right)

3.2. Genetic variant of the OPN4 gene and SAD

Global seasonality score (GSS) was assessed in 182 subjects of the previous cohort and failed to show any significant association with genotypes. However, individuals with the T/T genotype tended to have higher GSS (p=0.08) (**Figure 60**).

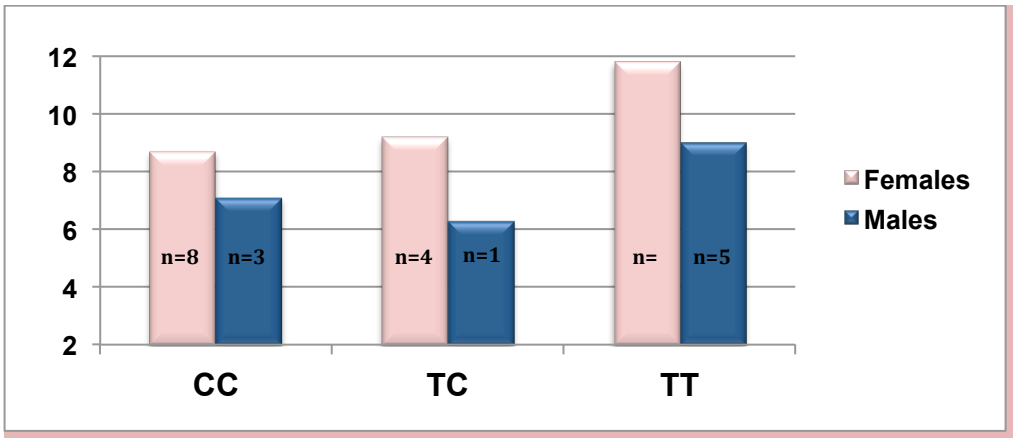


Figure 60. GSS in the three P10L genotypes

None of the genotypes revealed a significant association with the seasonal affective disorder (SAD) syndrome or the sub-syndrome (subSAD). Again, the TT genotype tended to be more represented in the (SAD) syndrome or the sub-syndrome (subSAD) groups (**Figure 61**).

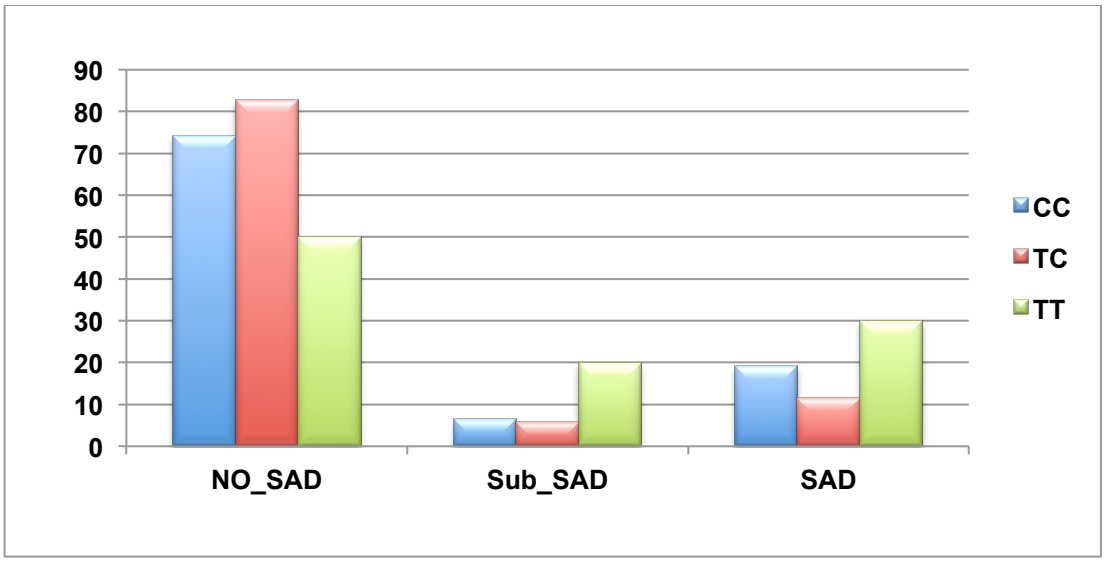


Figure 61. Distribution of the three P10L genotypes in the three categories (No Sad, Subsyndrome and SAD syndrome)

DISCUSSION

For the sake of clarity, the discussion is organized in three sections, which reflect the three projects of this PhD thesis.

5.1. Project 1- Melanopsin RGCs in hereditary optic neuropathies

The expansion of our initial investigation on mRGCs in hereditary optic neuropathies, after the two retinal specimens from Leber Hereditary Optic Neuropathy patients, involved an 87-year old dominant optic atrophy case. This patient had a terminal stage, very severe optic atrophy and demonstrated the highest rate of mRGCs sparing, reaching mRGCs/RGCs ratio of 48.6% (≅1% in controls; 3.1% in mild LHON and 38.5% in severe LHON cases) compared to 0.9% in the age-matched control (**Figure 20**). The distribution of mRGCs in the DOA case was flattened, whereas in the 85-year old control the peak in the macular region was maintained as previously shown for LHON patients and age-matched controls.

Two conclusions can be drawn by the overall results of this study. The main conclusion is that in two inherited optic neuropathies affecting selectively RGCs, the mRGCs system is relatively preserved, thus explaining the maintenance of the pupillary light reflex and the absence of sleep complaints in these blind patients. Our histological results are well supported by the normal melatonin suppression response after light exposure demonstrated in patients. A second conclusion concerns the first documentation that mRGCs may undergo reduction with age in humans, as shown by our morphometric evaluation in the control retinas. Furthermore, the distribution of mRGCs in the human retina peaks in the macular region. The DOA case represented an exception, possibly because of the combination of age and severe neurodegeneration. This study, including the LHON and DOA cases, has been published in the journal Brain (La Morgia et al., 2010) (see annex 1).

As an in-vivo follow-up study, we applied a recently described pupillometric method (Park et al., 2011) able to discriminate the contribution of mRGCs to the pupillary light reflex, to study a cohort of LHON affected patients, belonging to a single very large pedigree from Brazil (Sadun et al., 2003). We demonstrated that LHON patients maintained the pupillary light response to both red and blue light (Moura et al., 2011). However, both the amplitude of the pupil response and the ability to maintain a sustained response (mainly driven by mRGCs) were significantly reduced in LHON patients compared to controls. This reduced efficiency in initiating and maintaining the pupil constriction, suggests some loss of mRGCs compared to controls. In fact, the sustained response with blue light showed the most evident discrepancy between LHON and controls, pointing to a fast escape of the pupil due to the loss of mRGCs (Bremner, 2004). Interestingly, stratifying the patients according to the number of abnormal pupil parameters, we did not find any

correlation with visual function impairment, suggesting that the pupil function is dissociated at least in part from visual function in these patients. These findings are in line with previous observations demonstrating that both visual and pupil function are abnormal in LHON, but a relative sparing of the pupil afferent fibers is evident, comparing visual and pupil function (Bremner et al., 1999). Overall, these data are compatible with our previous histological results. In fact, mRGCs were relatively spared compared to the rate of loss of RGCs in optic neuropathy patients, but reduced as absolute number compared to age-matched controls (La Morgia et al., 2010). Thus, the pupillary reflex in LHON is present but less efficient when compared to controls. The only other study using a similar protocol in one monocular LHON case showed similar findings (Kawasaki et al., 2010). These studies leave open the question of how and why the mRGCs are resistant to the metabolic mitochondrial dysfunction compared to the regular RGCs.

To address this question we set an in-vitro investigation based on the hypothesis that the expression of the melanopsin photopigment might exert a protective role on mitochondrial dysfunction in mRGCs. We used a cell model enginerized to express melanopsin under the control of tetracycline. All experiments compared the same cells expressing or not this photopigment, under different conditions of light exposure and metabolic stress mimicking the mitochondrial dysfunction in LHON and DOA. To this end, cells were grown in the presence of increasing concentrations of rotenone, a classic mitochondrial complex I inhibitor, and in the presence of TBH, a pro-oxidant agent acting at the cytoplasmic level, and cell viability was measured by the SRB assay (Ghelli et al., 2008). These experiments were carried out in darkness, after white light and blue light exposure. Cell survival was not influenced by the expression of the melanopsin photopigment in none of the experimental conditions used. Within the limits of this set of experiments, our results indicate that melanopsin itself may not be responsible for the survival of mRGCs in mitochondrial optic neuropathies. A further candidate, which has not been explored in this project, is the neuropeptide pituitary adenilate cyclase-activating polypeptide (PACAP). This neuropeptide is selectively expressed by mRGCs and not by regular RGCs (Hannibal et al., 2002a, 2006a). Furthermore, there is evidence for its neuroprotective role in literature pointing to a possible role in mRGCs metabolic robustness.

A different point of view is related to the photoreceptive function of mRGCs, which makes them able to cope with the potentially harmful effect of light on cell viability. Light is a well known damaging agent on retinal cells, and RGCs, in particular, are the first retinal cell type exposed to incoming light. In LHON and DOA, under the condition of dysfunctional mitochondrial metabolism characterized by increased

ROS production, light might become a triggering factor for cell death adding to the mitochondrial impairment (Osborne et al., 2008). In this respect, mRGCs, despite carrying the same metabolic imbalance, are intrinsically protected by their photoreceptive properties, which are able to quench the damaging effects of light. This scenario has been debated in literature, but needs a solid experimental demonstration.

Finally, mRGCs robustness might be a more general phenomenon, beyond the mitochondrial metabolic dysfunction, as shown by a set of data from literature. For example, there is evidence that mRGCs survive to optic nerve transection, suggesting a general robustness of this cellular system (von Bussman et al., 1993; Robinson et al., 2004; Li et al., 2008). Remarkably, mRGCs are a photoreceptive system that predates the development of the eye as organ for vision and shares many similarities with the invertebrate photoreception (Davies et al., 2010). This very long evolutionary selection may underlie the present observations, leaving open the mechanisms implicated in their robustness in mitochondrial optic neuropathies.

5.2. Project 2- Optic nerve pathology, mRGCs and rest-activity circadian rhythm in AD, MCI and PD patients

5.2.1. OCT and rest-activity circadian rhythm in AD, MCI and PD patients

The first question we have addressed with this study concerned the occurrence of a significant optic neuropathy in common neurodegenerative disorders, such as AD and PD, and which pattern characterized this optic neuropathy. Our OCT results documented the occurrence of subclinical optic neuropathy in both disorders, with a clearly distinguishable pattern. In AD patients there was a significant reduction of RNFL thickness in the average measurements as well as in the superior and nasal quadrants (**Figure 26**). This reduction, however, was not evident when we considered only mild AD patients, being significant in the moderate subgroup. Opposite to this pattern, we documented in PD patients a reduction of RNFL thickness selectively limited to the temporal quadrant, and a close-to-significance reduction of the average measurements (**Figure 29**). The MCI individuals were unremarkable for all parameters measured by OCT compared to controls.

The existence of an optic neuropathy in AD patients has been histologically recognized since 1986 (Hinton et al., 1986) even if has been long debated in literature (Guo et al., 2010). In recent times, the use of OCT for in-vivo objective anatomical measurements of RNFL thickness prompted a number of studies in many neurodegenerative disorders including AD. Most studies reported a reduction in the average measurements (Parisi et al., 2011; Iseri et al., 2006; Paquet et al., 2007; Lu et

al., 2010; Kesler et al., 2011), as well as in the superior quadrant (Parisi et al., 2001; Iseri et al., 2006; Berisha et al., 2007; Lu et al., 2010; Kesler et al., 2011). Remarkably, none of these studies, including our current investigation, revealed an involvement of the temporal quadrant. Our results also point to RNFL thinning of the nasal quadrant, which has also been documented by a few of the above quoted reports (Parisi et al., 2001; Iseri et al., 2006). Overall, the subclinical optic neuropathy in AD seems to affect the magnocellular component of RGCs, as documented both histologically (Hinton et al., 1986; Sadun and Bassi, 1990) and more recently electrophysiologically (Sartucci et al., 2010). In this regard, it is of note that this pattern resembles that of glaucomatous optic neuropathy and, remarkably, there is a widely documented evidence of increased prevalence of glaucoma in AD patients (Bayer et al., 2002a,b; Tamura et al., 2006). The limit distinguishing glaucoma from AD-related optic neuropathy is blurry and there is a consistent possibility that optic nerve involvement in AD is characterized by a continuous spectrum of severity ranging from a subclinical to a glaucoma-like optic neuropathy. The pattern followed by this neurodegenerative process affecting RGCs is the same. In this context our results are coherent with the current knowledge on AD optic neuropathy and set for the hypothesis we are pursuing in this project of a possible co-involvement of mRGCs in AD.

Our inconclusive results with MCI individuals are in contrast with some reports documenting a reduction of RNFL thickness also in MCI (Paquet et al., 2007, Kesler et al., 2011). There are multiple possible reasons for our failure to observe such a reduction: first, the small sample size of our cohort, and secondly, we did not include only the amnesic variant of MCI, the most predictive for conversion to AD (Fischer et al., 2007).

In PD patients our OCT study also revealed a subclinical optic neuropathy, affecting significantly only the nerve fibers entering the temporal quadrant of the optic disc. The contralateral eye was more affected than the ipsilateral to the most affected body side, supporting an asymmetry of the neurodegenerative process that involves both the substantia nigra and the eye on the same side. Similarly, an asymmetric impairment has been reported for the olfactory function, further supporting a general asymmetry in the neuropathological process in PD (Zucco et al., 2001). These results identified in PD patients a pattern of axonal loss typically seen in LHON and DOA, where the temporal fibers belonging to the papillo-macular bundle are characteristically susceptible (Carelli et al., 2004). Both diseases are associated with a complex I defect (Carelli et al., 2007, 2009), which is also recognized as a key feature in the pathogenesis of PD, either in the sporadic cases as well as in the

genetic forms (Schapira et al, 2008; Whithworth et al., 2009), linking the current results with the pattern observed in LHON and DOA. Previously, other authors documented optic nerve pathology in PD, some reporting a preferential loss of fibers in the infero-temporal quadrants, consistent with the involvement of the papillo-macular bundle (Inzelber et al., 2004; Yavas et al., 2007; Moschos et al., 2011). Macular and foveal abnormalities have also been documented (Hajee et al., 2009). However, Altintas and coauthors reported a significant RNFL thinning of the superior and nasal quadrants (Altintas et al., 2008), whereas Archibald and coauthors failed to find any differences between PD and controls (Archibald et al., 2011b). The role of a specific PD neuropathology in the retina is controversial and clear evidence of alfa-synuclein deposition is still missing. The most consistent link we hypothesize between the subclinical optic nerve involvement in PD and the major mitochondrial optic neuropathies LHON and DOA is based on complex I dysfunction and altered mitochondrial dynamics (Carelli et al., 2004). This latter link is highlighted by mitochondrial network fragmentation in patient-derived cells from both DOA and PD affected individuals carrying respectively OPA1 and PINK1/Parkin mutations (Carelli et al., 2004; Schapira et al., 2008; Withworth et al., 2009). The balance between mitochondrial fission and fusion seems crucial for the correct execution of mitochondrial quality control and excessive mitophagy has been documented in RGCs of OPA1 mutant mouse leading to their degeneration, as well as it is now a central theme in PD pathogenesis (Withworth et al., 2009; White et al., 2009; Vives-Bauza et al., 2011).

Overall, all our results on RNFL thickness in AD, MCI, PD and controls were correlated to the aging process, confirming previous studies at both histological and OCT level (Johnson et al., 1987; Balazsi et al., 1984, Feuer et al., 2011; Budenz et al., 2007). Thus, a neurodegenerative process affects, with the documented differences in pattern, both AD and PD patients in an age-related fashion supporting the possibility that mRGCs could be involved.

The second question we addressed with this study concerned the occurrence of rest-activity circadian rhythm abnormalities in AD, MCI and PD patients compared to age-matched controls. We used a prolonged actigraphic monitoring to assess the interdaily stability, intradaily variability and relative amplitude of the rest-activity circadian rhythms applying non-parametric methods (Witting et al., 1990; Van Someren et al., 1999). We also derived, by these recordings, sleep minutes and sleep efficiency. As a general observation, both categories of AD and PD patients displayed a wide variability for all the parameters measured, possibly reflecting a heterogeneity of the circadian disturbances in these diseases (**Figures 33 and 34**). In

particular, AD patients showed a tendency towards an increased intradaily variability and reduced relative amplitude of the rest-activity circadian rhythm. Furthermore, AD patients tended to be less active during the day. PD patients revealed a significantly increased intradaily variability, reduced relative amplitude of rest-activity circadian rhythm and increased activity during the resting period compared to controls. MCI individuals were not significantly different from controls for any of the parameters measured.

Most of the studies reporting the occurrence of circadian dysfunction in AD included moderate or severe AD patients, documenting a generalized and more relevant circadian dysfunction than in our mild-moderate case series (Satlin et al., 1995; Witting et al., 1990; Hatfield et al., 2004; Van Someren et al., 1996; Harper et al., 2008; Volicer et al., 2001). Previous studies investigating mild AD patients failed to reveal significant abnormalities (Van Someren et al., 1996; Hatfield et al., 2004). However, studies using laboratory-based polysomnography showed reduced sleep efficiency and amount of slow wave sleep, even in the mild cases (Prinz et al., 1982; Moe et al., 1995). Similarly, in our series of mild-moderate cases we found reduced sleep efficiency compared to controls. Moreover, in many of the case series reported, a broad range of activity pattern was also evident in subjects suffering moderate dementia, with some patients showing a pattern resembling that of controls and others with severely compromised behavior (Hatfield et al., 2004). Interestingly, Hatfield and coauthors conducted a 1-year follow-up study in mild-moderate AD patients, showing that the actigraphic pattern did not deteriorate at follow-up, even if there was a significant decrease in cognitive functions (Hatfield et al., 2004). This suggests that cognitive and circadian abnormalities behave with a different rate of progression and may be sustained by different pathophysiological mechanism. Further, in this case series the patients with a poor outcome in terms of instituzionalization or severe deterioration of cognitive functions had a significantly lower stability of circadian parameters at baseline evaluation (Hatfield et al., 2004). Compatibly with these observations, we also documented that AD patients with the most severely affected circadian parameters presented the worst outcome with one patient being instituzionalized and one rapidly deteriorating (data not shown). In other words, the presence of significant circadian dysfunction in the early stage of disease may be a predictive factor of poor outcome in patients with AD. Thus, our failure to show significant differences between AD patients and controls may depend on the inclusion of only mild-moderate cases and on the heterogeneity of circadian-impaired patients at a group level. We were constrained in selecting the mild-moderate AD patients because of the need for compliance to carry out both OCT and

ophthalmological examinations. Other limitations intrinsic to this study may be represented by the overall small sample size of this cohort and the non-controlled conditions in terms of season of actigraphic recordings, light exposure and meal-time due to the difficulties of studying these patients in a more controlled laboratory setting. Moreover, the duration of our actigraphic monitoring may be not fully sufficient to detect abnormalities of circadian parameters (Van Someren, 2007). Actigraphic parameters did not correlate with clinical data (disease duration and severity of cognitive impairment) or OCT data in the overall group of AD patients. However, selecting only patients with at least one abnormal circadian parameter, we demonstrated that IV significantly correlated with average, superior and nasal RNFL thickness at OCT evaluation (**Figure 36**). This correlation is particularly significant for our hypothesis, supporting the possible involvement of retinal pathology, i.e. loss of mRGCs, in the pathogenesis of circadian dysfunction.

Concerning the PD patients, our actigraphic investigation documented a significant increase of the intradaily variability, reduction of relative amplitude and increased activity during the resting period. These findings are concordant with those reported by Whitehead and coauthors in a cohort of 50 patients (Whitehead et al., 2008). Only another study assessed the rest-activity circadian rhythm in PD patients, showing a significant reduction of the circadian rhythm pattern evaluated with a different methodology (Niwa et al., 2011). Further, the finding of increased activity during the resting period is concordant with other actigraphic studies in PD (Van Hilten et al., 1984; Stavitsky et al., 2010). The interpretation of actigraphic results in PD patients must take into account the possible role of confounding factors such as the motor disability, the circadian fluctuations of motor functions and the influence of dopaminergic drugs (Whitehead et al., 2008; Bruguerolle et al., 2002). However, Vezoli and coauthors demonstrated in a MPTP-monkey model of PD that circadian dysfunction occurs early, before the appearance of motor symptoms, suggesting that this may be a pathological feature independent from the evolution of motor disability (Vezoli et al., 2011). Furthermore, a recent Drosophila model of PD expressing α-synuclein mutants recapitulated non-motor symptoms including abnormal sleep-like behavior, altered locomotor activity and abnormal circadian periodicity, all preceding the onset of motor symptoms (Gajula Balija et al., 2011). Compatibly with these evidences from animal models of PD we failed to correlate our actigraphic results with both duration and severity of motor symptoms. Moreover, there is evidence that dopamine depletion per se may be relevant for the occurrence of circadian impairment (Boulamery et al., 2010; Gravotta et al., 2011). This might be particulary important if we consider that dopamine depletion has been demonstrated in PD

retinas (Harnois and Di Paolo, 1990) and that a direct interaction of retinal dopaminergic amacrine cells with mRGCs has also been shown (Zhang et al., 2008; Vugler et al., 2007).

We also documented a significant reduction of sleep minutes and sleep efficiency in PD patients and of sleep efficiency in AD patients, as previously reported (Stavitsky et al., 2012; Yesavage et al., 2004). These findings are in accordance with many reports documenting the occurrence of sleep disturbances in PD and AD (Menza et al., 2010; Iranzo et al., 2011; Wu and Swaab, 2007; Tractenberg et al., 2006)

At variance with others, we did not find any difference in terms of sleep questionnaires assessing excessive daytime sleepiness and subjective quality of sleep between AD, PD patients and controls. This result may be explained by the small sample size of our cohort and the wide heterogeneity in the questionnaires scores.

5.2.3. Melanopsin RGCs and optic nerve studies in post-mortem specimens from AD cases and controls

We analyzed the post-mortem retinal specimens and corresponding optic nerve cross-sections of 11 neuropathologically definite AD patients and 11 age-matched controls. Looking at the overall results, we documented a significant loss of mRGCs in AD retinas compared to controls and a close-to-significance loss of axons in optic nerve cross-sections, and thus of RGCs. Consequently, the mRGCs/RGCs ratio was similar for AD cases and controls (**Figures 48-50**). These results may apparently suggest that, to some extent, the neurodegenerative process in AD affects in parallel and at similar rate both mRGCs and RGCs. The observation of axonal loss in AD cases just confirms the previous notion in literature that optic neuropathy can be found in AD patients (Guo et al., 2010; Hinton et al., 1986; Sadun and Bassi, 1990), further evidenced in this project by the OCT results. Qualitatively, the axonal loss in the optic nerve specimens of the most severe cases affected the large axons of the supero-nasal and inferior sectors, with sparing of the papillomacular bundle temporal fibers (**Figure 46**), substantiating at histopathological level the pattern observed at OCT (**Figure 26**). This pattern is coherent with that seen in glaucoma, supporting the argument that in AD patients it might be a spectrum of optic neuropathy continuum ranging from mild axonal loss to glaucoma-like cases. This may explain the high prevalence of glaucoma in AD patients (Bayer et al., 2002).

Axonal loss in controls is an age-dependent phenomenon (**Figure 51**). This is beautifully shown by the progressive loss of axons over the decades (**Figure 52**), which again confirms observations done more than 20 years ago (Dolman et al., 1980; Balazsi et al., 1984; Johnson et al., 1987). Our results on AD patients also indicate a similar age-related dependence of axonal loss but with a sharper decline

after 80s. We interpret this as the additional effect of aging and neurodegeneration affecting RGCs in AD patients. Similar results correlating the axonal loss to age were obtained by our OCT studies in both controls and AD patients (**Figure 27**).

Concerning the mRGCs population, this is also lost in an age-related fashion in controls, as we previously reported (La Morgia et al., 2010) (**Figure 51**). Intriguingly, in AD cases we found that mRGCs were remarkably reduced in density at a similar degree across decades (**Figure 52**). This finding suggests that mRGCs undergo a neurodegenerative process, which most probably is independent from the axonal loss, or in other words from the optic neuropathy. In fact, mRGCs density reduction occurs also in patients with no axonal loss and normal optic nerve (60s and 70s). This leads to an mRGCs/RGCs ratio inferior to 1% in AD patients younger than 80 years, whereas this ratio rises to 1.5% in patients older than 80 years, and over 2% in the 2 cases with the most severe axonal loss (see **Figure 53**).

Thus, compared to controls, the patterns of axonal and mRGCs loss, as calculated by z-scores, is profoundly different (**Figure 62**), suggesting that for mRGCs there is a distinctive neurodegenerative phenomenon, part of the Alzheimer neuropathology, whereas for RGCs there is the overlapping of AD neuropathology and aging.

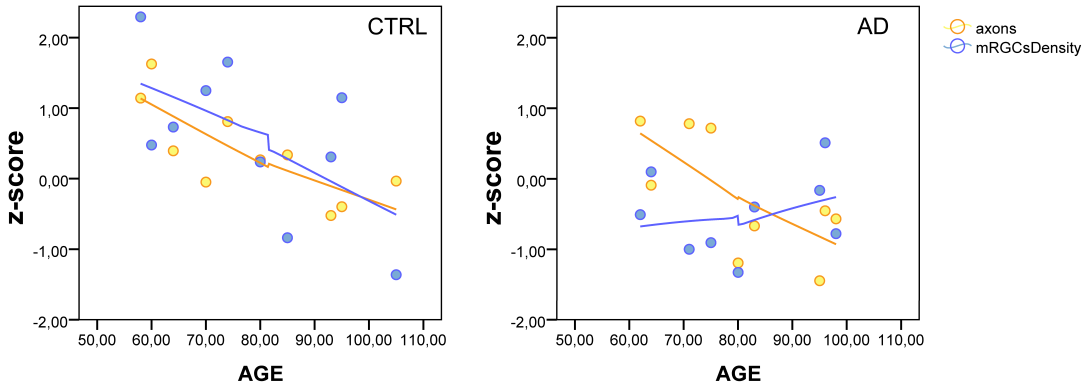


Figure 62. Pattern of axonal and mRGCs loss in controls and AD patients

In support to the above conclusions, the morphology of mRGCs qualitatively showed an increased amount of fragmented dendrites, which may be a hallmark of a neurodegenerative process that specifically affects this population of cells. The evidence that mRGCs may be affected by the neurodegenerative process of AD, independently from age, possibly explains the occurrence of circadian rhythm dysfunction documented by some authors also in the early stage of the disease (Hu et al., 2009; Sterniczuk et al., 2010).

Interestingly, mRGCs density correlated with the severity of AD pathology at the level of superior-middle temporal cortex and lateral geniculate body of the

hippocampus, suggesting that the degree of mRGCs loss might be an index of the severity of the disease.

Considering the entire project 2 we can conclude that OCT, actigraphic recordings and histopathological studies indicate that the visual system is consistently affected in a subgroup of AD patients. These are possibly the patients suffering more extensively circadian disturbances. In fact, when we isolated the "circadian-impaired" patients based on the actigraphic parameters, we have been able to highlight a correlation with the OCT retinal measurements in the same patients.

5.3. Project 3-OPN4 polymorphism, chronotype and seasonal affective disorder

The third project of this PhD thesis was focused on the previous report that genetic variation in the *OPN4* gene, encoding for the melanopsin photopigment, was associated with SAD (Roecklein et al., 2009). Based on the role of mRGCs in circadian photoentrainement and the recent data on their involvement in regulation of sleep homeostasis, and knowing that different chronotypes have a different homeostatic regulation of sleep, we evaluated a possible association of the same missense P10L variant with chronotype.

We failed to show any significant association between this polymorphic variant and chronotypes. Genetic determination of chronotypes has already been investigated, by studying clock genes, with heterogeneous and frequently non-reproduceable results (Wulff et al., 2009). A possible confounding factor is represented by age. We also considered the subgroup of patients younger than 40 years, again without finding any significant association between genotypes of the P10L variant and chronotype.

This investigation is the first to address the possibility that genetic variants in the *OPN4* gene may be influential on sleep and we focused in particular on the chronotype. Association studies need very large cohorts to reach statistical power and the current study might just not be sufficient to highlight a positive association. In fact, the TT genotype of the P10L variant is present at a very low frequency in the general population and this implies that a very large cohort of individuals should be analyzed to reach a solid statistical significance. Furthermore, our cohort was also umbalanced in terms of gender, with a prevalent representation of females. Last, we screened only one polymorphic variant of the *OPN4* gene based on its previous association with SAD (Roeckelein et al., 2009), but other possible candidate SNPs may be further investigated.

In a subgroup of this cohort we also had available SPAQ questionnaires evaluating the possible occurrence of symptoms suggestive of the SAD syndrome. Interestingly, even if not significant, we found a tendency for an association between the TT

genotype with SAD and subSAD. Individuals with TT genotype tended to have also a higher GSS score, in particular females. Within the limits of a non-significant tendency, this finding is supporting the initial results by Roecklein and coauthors in a second independently collected cohort (Roecklein et al., 2009). A major difference between our study and that of Roecklein and coauthors resides in the definition of SAD, which was based on a psychiatric diagnosis in their study, whereas our cohort was investigated only by means of questionnaires.

However, the concordance of our results with the previous study is particularly intriguing if we consider the strong circadian basis of SAD, the abnormal hypothalamic response to light (Vandewalle et al., 2011b) and the beneficial effect of light therapy, and in particular of blue light, demonstrated in these patients (Glickman et al., 2006).

CONCLUSIONS AND FUTURE DIRECTIONS

The three projects of this PhD thesis have as a common theme the role played by the newly recognized photoreceptors in the retina, the retinal ganglion cells expressing the photopigment melanopsin (mRGCs), in driving photoentrainment of circadian rhythms. The three projects undertook investigations exploring different issues in human pathologies associated by the link of optic nerve pathology and circadian dysfunction of rest-activity rhythm, hypothesizing the causal involvement of mRGCs (for a review see La Morgia et al., 2012a).

With the first project, we made the remarkable observation that in retinal and optic nerve specimens from patients affected by inherited mitochondrial optic neuropathies (LHON and DOA), which selectively affect retinal ganglion cells (RGCs), the subpopulation of mRGCs was relatively spared. These results explained some clinical features of these blind patients such as the maintenance of the melatonin suppression response induced by light, the absence of subjective sleep complaints and, to some degrees, the maintenance of the pupillary light reflex. To better define in vivo this latter issue, we applied the innovative protocol of pupillometric evaluation distinguishing the mRGCs-driven sustained response to blue light from the transient response evoked by red light. By this mean we studied LHON affected patients documenting how they may range from perfectly normal pupillary responses to partially reduced responses, characterized by the phenomenon of pupillary escape. We then asked the question if the mechanisms underlying mRGCs resistance to mitochondrial dysfunction resided in the main biological, unique, feature of these cells, i.e. the expression of the photopigment melanopsin. In a series of experiments that took advantage of a cellular model where melanopsin expression could be driven by tetracycline, we studied different stress models, under different light conditions, failing to find that melanospin expression could represent a protective factor to these metabolic insults. Thus, the question of what makes so robust these ancient photoreceptors, which evolved before the development of the eye as an organ for vision, remains open. These findings underline the long selection for a fundamental function in life, the photoentrainment of circadian rhythms. In the next future, more studies will be needed to fully characterize the metabolic properties of mRGCs. In particular, fields of investigation will involve handling of calcium fluxes in these cells, as well as the overall mitochondrial network characteristics and maintenance, the role played by other factor exclusively expressed by mRGCs such as PACAP, and lastly the role played by light in damaging retinal cell components.

The second project investigated the two most frequent age-related neurodegenerative disease, Alzheimer and Parkinson disease. The hypothesis, in this case, was that in both disorders there is evidence of optic neuropathy and circadian dysfunction. We thought to link these two features hypothesizing that the mRGC neuronal system could be affected. We first characterized the optic neuropathy in both AD and PD patients by optical coherence tomography, which allows quantitative assessment of retinal and optic nerve head anatomy. We confirmed that both AD and PD patient present different degrees of optic neuropathy, evidencing a sharply distinguishable pattern. AD optic neuropathy is characterized by a glaucoma-like pattern with loss of large axons as reflected by the reduction of RNFL thickness in the supero-nasal quadrants at OCT. Opposite to AD, the PD optic neuropathy is characterized by the exclusive loss of the small axons in the papillomacular bundle, as reflected by the temporal loss of RNFL thickness. We second characterized, by means of prolonged actigraphic recordings, the rest-activity circadian rhythm in AD and PD patients, in order to assess the circadian dysfunction in these patients. In both diseases we identified a wide variability, which hampered the possibility to obtain clearly significant results in the overall group of AD patients. However, it seems clear that a subgroup of AD, as well as PD patients can be selected for having at least one of the circadian parameters measured significantly abnormal. Interestingly, the subgroup of "circadian-impaired" AD patients revealed a correlation between the parameters of circadian dysfunction and those of optic neuropathy, as assessed by OCT. The third approach, limited to AD, was to thoroughly investigate directly the mRGC system in post-mortem retinal and optic nerve specimens, following our protocol established in studying mitochondrial optic neuropathies. This approach has been revealing by showing that mRGCs are lost in a fashion independent from that of the optic neuropathy, which seems mostly a mixture of an age-related process overlapped by the neurodegeneration. The reduction in density of mRGCs did not obey the age-dependance, being present across all decades, from 60s to 90s, with similar magnitude. Considering that we also qualitatively observed frequent abnormalities of the mRGCs morphology, in particular fragmentation of their dendritic arborization, we may conclude that the overall mRGC system is affected and possibly contributes to the genesis of the circadian dysfunction in AD.

These results generate many questions and future projects to elucidate the involvement of mRGCs in AD. For example, what is the role of amyloid deposition in the retina? It would be interesting to explore if a specific subclass of mRGCs is specifically involved in AD. To this end the use of flat-mounted retinal preparations

may be of great help, by studying the dendritic morphology, considering the abnormalities observed in this study. Furthermore, is there a corresponding recognizable pathology affecting the SCN in hypothalamus? Similar to our approach in mitochondrial optic neuropathies, the mRGCs could be also explored in vivo by light-controlled pupillometry applied to different neurodegenerative disorders for which there is evidence of circadian dysfunction. Another possibility is to investigate the mRGCs system using functional MRI paradigms, as previously reported (Vandewalle et al., 2009a). Further, given the role of mRGCs in regulating the homeostatic process in sleep, another possible research field is the detailed characterization of sleep homeostasis in AD. The accurate assessment of circadian dysfunction in neurodegeneration and aging is of particular importance if we consider that the presence of abnormal circadian rhythm activity has been recognized as a strong predictor for the development of dementia (Tranah et al., 2011). To this end, the follow-up study of MCI subjects, in particular the amnesic subgroup, will be of special interest, possibly validating visual/circadian parameters as predictors of conversion to dementia. Further, the possibility to counteract the impairment of the system deputed to circadian photoentrainment by light-therapy and other chronobiological approaches is of extreme importance in this context (Zhou et al., 2012).

The entire set of histopathological studies that we carried out in AD, is also planned for PD patients, following the same experimental design. For PD patients we will also verify the possible presence of α-synuclein deposition in the retina, as a specific marker of neurodegeneration, in the context of mRGCs/RGCs system.

Another possible intriguing scenario will be the characterization of the mRGCs system in normal aging, and in particular in centenarians.

The last project of this PhD thesis addressed the possible association between melanopsin gene variants, human chronotypes and seasonal affective disorder. We did not find a significant association between the previously reported missense variant of the *OPN4* gene with chronotype, whereas a partial confirmation for the association with SAD was found. However, the investigation of other variants of the *OPN4* gene in conjunction with the evaluation of other clock genes might be planned for the next future in a more homogeneous and large cohort, together with other functional measurements, such as slow wave activity during sleep, pupillometric and functional magnetic resonance studies with different paradigms of light stimulation in different chronotypes and in circadian rhythm sleep disorders, such as delayed and advanced sleep phase syndrome.

REFERENCES

Aaker GD, Myung JS, Ehrlich JR, Mohammed M, Henchcliffe C, Kiss S. Detection of retinal changes in Parkinson's disease with spectral-domain optical coherence tomography. *Clin Ophthalmol* 2010;4:1427-32.

Achermann P, Borbély AA. Mathematical models of sleep regulation. *Front Biosci* 2003; 8: s683-693.

Adler CH, Thorpy MJ. Sleep issues in Parkinson's disease. *Neurology* 2005; 64: s12-20.

Altimus CM, Güler AD, Villa KL, McNeill DS, Legates TA, Hattar S. Rods-cones and melanopsin detect light and dark to modulate sleep independent of image formation. *Proc Natl Acad Sci U S A* 2008; 105:19998-20003.

Altintaş O, Işeri P, Ozkan B, Cağlar Y. Correlation between retinal morphological and functional findings and clinical severity in Parkinson's disease. *Doc Ophthalmol* 2008;116: 137-146.

Ancoli-Israel S, Poceta JS, Stepnowsky C, Martin J, Gehrman P. Identification and treatment of sleep problems in the elderly. *Sleep Med Rev* 1997; 1:3-17.

Archer SN, Robilliard DL, Skene DJ, Smits M, Williams A, Arendt J, et al. A length polymorphism in the circadian clock gene Per3 is linked to delayed sleep phase syndrome and extreme diurnal preference. *Sleep* 2003; 26: 413-5.

Archibald NK, Clarke MP, Mosimann UP, Burn DJ. The retina in Parkinson's disease. *Brain* 2009; 132: 1128-45.

Archibald NK, Clarke MP, Mosimann UP, Burn DJ. Visual symptoms in Parkinson's disease and Parkinson's disease dementia. *Mov Disord* 2011a;26:2387-95.

Archibald NK, Clarke MP, Mosimann UP, Burn DJ. Retinal thickness in Parkinson's disease. *Parkinsonism Relat Disord* 2011b;17:431-6.

Arnulf I. Excessive daytime sleepiness in parkinsonism. *Sleep Med Rev* 2005; 9: 185-200.

Auger RR, Boeve BF. Sleep disorders in neurodegenerative diseases other than Parkinson's disease. *Handb Clin Neurol* 2011; 99: 1011-1050.

Balazsi AG, Rootman J, Drance SM, Schulzer M, Douglas GR. The effect of age on the nerve fiber population of the human optic nerve. *Am J Ophthalmol* 1984;97:760-6.

Barboni P, Savini G, Valentino ML, Montagna P, Cortelli P, De Negri AM, et al. Retinal nerve fiber layer evaluation by optical coherence tomography in Leber's hereditary optic neuropathy. *Ophthalmology* 2005;112:120-126.

Barboni P, Carbonelli M, Savini G, Ramos Cdo V, Carta A, Berezovsky A, et al. Natural history of Leber's hereditary optic neuropathy: longitudinal analysis of the retinal nerve fiber layer by optical coherence tomography. *Ophthalmology* 2010;117:623-627.

Baver SB, Pickard GE, Sollars PJ, Pickard GE. Two types of melanopsin retinal ganglion cell differentially innervate the hypothalamic suprachiasmatic nucleus and the olivary pretectal nucleus. *Eur J Neurosci* 2008; 27: 1763-1770.

Bayer AU, Ferrari F, Erb C. High occurrence rate of glaucoma among patients with Alzheimer's disease. *Eur Neurol* 2002a; 47: 165-168.

Bayer AU, Keller ON, Ferrari F, Maag KP. Association of glaucoma with neurodegenerative diseases with apoptotic cell death: Alzheimer's disease and Parkinson's disease. *Am J Ophthalmol* 2002b; 133: 135-137.

Beaulieu-Bonneau S, Hudon C. Sleep disturbances in older adults with mild cognitive impairment. *Int Psychogeriatr*. 2009;21:654-66.

Ben V, Bruguerolle B. Effects of bilateral striatal 6-OHDA lesions on circadian rhythms in the rat: a radiotelemetric study. *Life Sci* 2000; 67: 1549-1558.

Benarroch EE. The melanopsin system: Phototransduction, projections, functions, and clinical implications. *Neurology* 2011;76:1422-7.

Berisha F, Feke GT, Trempe CL, McMeel JW, Schepens CL. Retinal abnormalities in early Alzheimer's disease. *Invest Ophthalmol Vis Sci* 2007; 48: 2285-2289.

Berson DM, Dunn FA, Takao M. Phototransduction by retinal ganglion cells that set the circadian clock. *Science* 2002; 295: 1070-1073.

Berson DM. Strange vision: ganglion cells as circadian photoreceptors. *Trends Neurosci* 2003; 26: 314-320.

Blanks JC, Hinton DR, Sadun AA, Miller CA. Retinal ganglion cell degeneration in Alzheimer's disease. *Brain Res* 1989; 501: 364-372.

Blanks JC, Torigoe Y, Hinton DR, Blanks RH. Retinal pathology in Alzheimer's disease. I. Ganglion cell loss in foveal/parafoveal retina. *Neurobiol Aging* 1996a; 17: 377-384.

Blanks JC, Schmidt SY, Torigoe Y, Porrello KV, Hinton DR, Blanks RH. Retinal pathology in Alzheimer's disease. II. Regional neuron loss and glial changes in GCL. *Neurobiol Aging* 1996b; 17: 385-395.

Bliwise DL, Ansari FP, Straight LB, Parker KP. Age changes in timing and 24-hour distribution of self-reported sleep. *Am J Geriatr Psychiatry* 2005;13:1077-1082.

Bodis-Wollner I, Yahr MD. Measurements of visual evoked potentials in Parkinson's disease. *Brain* 1978; 101: 661-671.

Bodis-Wollner I, Marx MS, Mitra S, Bobak P, Mylin L, Yahr M. Visual dysfunction in Parkinson's disease. Loss in spatiotemporal contrast sensitivity. *Brain* 1987; 110: 1675-1698.

Bodis-Wollner I. Visual deficits related to dopamine deficiency in experimental animals and Parkinson's disease patients. *Trends Neurosci* 1990; 13: 296-302.

Bodis-Wollner I. Retinopathy in Parkinson Disease. *J Neural Transm* 2009; 116: 1493-501.

Boeve BF, Silber MH, Saper CB, Ferman TJ, Dickson DW, Parisi JE, et al. Pathophysiology of REM sleep behaviour disorder and relevance to neurodegenerative disease. *Brain* 2007; 130: 2770-2788.

Borbély AA. A two process model of sleep regulation. *Hum Neurobiol* 1982; 1: 195-204.

Borbély AA, Tobler I. Manifestations and functional implications of sleep homeostasis. In: Montagna P, Chockroverty S, editors. Handbook of clinical neurology, vol. 98 (3rd series). Sleep disorders: Part 1. Amsterdam: Elsevier 128 *Science*; 2011. p. 205–213.

Bordet R, Devos D, Brique S, Touitou Y, Guieu JD, Libersa C, et al. Study of circadian melatonin secretion pattern at different stages of Parkinson's disease. *Clin Neuropharmacol* 2003; 26: 65-72.

Boulamery A, Simon N, Vidal J, Bruguerolle B. Effects of L-Dopa on circadian rhythms of 6-OHDA striatal lesioned rats: a radiotelemetric study. *Chronobiol Int* 2010;27:251-64.

Braak H, Del Tredici K, Bratzke H, Hamm-Clement J, Sandmann-Keil D, Rüb U. Staging of the intracerebral inclusion body pathology associated with idiopathic Parkinson's disease (preclinical and clinical stages). *J Neurol* 2002; 249 Suppl 3:III/1-5.

Brainard GC, Richardson BA, King TS, Matthews SA, Reiter RJ. The suppression of pineal melatonin content and N-acetyltransferase activity by different light irradiances in the Syrian hamster: a dose-response relationship. *Endocrinology* 1983; 113: 293-296.

Brainard GC, Hanifin JP, Greeson JM, Byrne B, Glickman G, Gerner E, et al. Action spectrum for melatonin regulation in humans: evidence for a novel circadian photoreceptor. *J Neurosci* 2001; 21: 6405-6412.

Bremner FD, Shallo-Hoffmann J, Riordan-Eva P, Smith SE. Comparing pupil function with visual function in patients with Leber's hereditary optic neuropathy. *Invest Ophthalmol Vis Sci* 1999;40:2528-34.

Bremner FD. Pupil assessment in optic nerve disorders. *Eye* (Lond). 2004;18:1175-81.

Brown TM, Gias C, Hatori M, Keding SR, Semo M, Coffey PJ, et al. Melanopsin contributions to irradiance coding in the thalamo-cortical visual system. *PLoS Biol* 2010; 8: e1000558.

Bruguerolle B, Simon N. Biologic rhythms and Parkinson's disease: a chronopharmacologic approach to considering fluctuations in function. *Clin Neuropharmacol* 2002; 25: 194-201.

Brusini P, Johnson CA. Staging functional damage in glaucoma: review of different classification methods. *Surv Ophthalmol* 2007; 52: 156-179.

Budenz DL, Anderson DR, Varma R, Schuman J, Cantor L, Savell J, et al. Determinants of normal retinal nerve fiber layer thickness measured by Stratus OCT. *Ophthalmology* 2007;114:1046-52.

Bulens C, Meerwaldt JD, Van der Wildt GJ, Van Deursen JB. Effect of levodopa treatment on contrast sensitivity in Parkinson's disease. *Ann Neurol* 1987; 22: 365-9

Buysse DJ, Reynolds CF 3rd, Monk TH, Berman SR, Kupfer DJ. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. *Psychiatry Res* 1989;28:193-213.

Cagnacci A, Bonuccelli U, Melis GB, Soldani R, Piccini P, Napolitano A, et al. Effect of naloxone on body temperature in postmenopausal women with Parkinson's disease. *Life Sci* 1990; 46: 1241-1247.

Cai Y, Liu S, Sothern RB, Xu S, Chan P. Expression of clock genes Per1 and Bmal1 in total leukocytes in health and Parkinson's disease. *Eur J Neurol* 2010;17:550-4.

Cajochen C, Münch M, Knoblauch V, Blatter K, Wirz-Justice A. Age-related changes in the circadian and homeostatic regulation of human sleep. *Chronobiol Int* 2006; 23: 461-474.

Cajochen C, Chellappa S, Schmidt C. What keeps us awake?--the role of clocks and hourglasses, light, and melatonin. *Int Rev Neurobiol* 2010; 93: 57-90.

Carelli V, Ross-Cisneros FN, Sadun AA. Mitochondrial dysfunction as a cause of optic neuropathies. *Prog Retin Eye Res* 2004;23:53-89.

Carelli V, La Morgia C, Iommarini L, Carroccia R, Mattiazzi M, Sangiorgi S, et al. Mitochondrial optic neuropathies: how two genomes may kill the same cell type? *Biosci Rep* 2007;27:173-84.

Carelli V, La Morgia C, Valentino ML, Barboni P, Ross-Cisneros FN, Sadun AA. Retinal ganglion cell neurodegeneration in mitochondrial inherited disorders. *Biochim Biophys Acta* 2009;1787:518-28.

Carpenter BD, Strauss ME, Patterson MB. Sleep disturbances in community-dwelling patients with Alzheimer's disease. *Clin Gerontol* 1995; 16: 35–49.

Carvalho-Bos SS, Riemersma-van der Lek RF, Waterhouse J, Reilly T, Van Someren EJ. Strong association of the rest-activity rhythm with well-being in demented elderly women. *Am J Geriatr Psychiatry* 2007; 15: 92-100.

Chambille I, Serviere J. Neurotoxic effects of neonatal injections of monosodium L-glutamate (L-MSG) on the retinal ganglion cell layer of the golden hamster: Anatomical and functional consequences on the circadian system. *Journal of Comparative Neurology* 1993; 338: 67–82.

Chakravarthy U, Evans J, Rosenfeld PJ. Age-related macular degeneration. *BMJ* 2010; 340: c981.

Chaudhuri KR, Pal S, DiMarco A, Whately-Smith C, Bridgman K, Mathew R, et al. The Parkinson's disease sleep scale: a new instrument for assessing sleep and nocturnal disability in Parkinson's disease. *J Neurol Neurosurg Psychiatry* 2002;73:629-35.

Chokroverty S. Sleep and degenerative neurologic disorders. *Neurol Clin* 1996; 14: 807-826.

Claassen DO, Josephs KA, Ahlskog JE, Silber MH, Tippmann-Peikert M, Boeve BF. REM sleep behavior disorder preceding other aspects of synucleinopathies by up to half a century. *Neurology* 2010; 75: 494-499.

Coben LA, Danziger WL, Hughes CP. Visual evoked potentials in mild senile dementia of Alzheimer type. *Electroencephalogr Clin Neurophysiol* 1983; 55: 121-130.

Compta Y, Santamaria J, Ratti L, Tolosa E, Iranzo A, Muñoz E, et al. Cerebrospinal hypocretin, daytime sleepiness and sleep architecture in Parkinson's disease dementia. *Brain* 2009; 132: 3308-17.

Cooke JR, Loredo JS, Liu L, Marler M, Corey-Bloom J, Fiorentino L, et al. Acetylcholinesterase inhibitors and sleep architecture in patients with Alzheimer's disease. *Drugs Aging* 2006; 23: 503-511.

Cooper HM, Herbin M, Nevo E. Ocular regression conceals adaptive progression of the visual system in a blind subterranean mammal. *Nature* 1993;361:156-9.

Cubo E, Tedejo RP, Rodriguez Mendez V, López Peña MJ, Trejo Gabriel Y, Galán JM. Retina thickness in Parkinson's disease and essential tremor. *Mov Disord* 2010;25:2461-2.

Curcio CA, Allen KA. Topography of ganglion cells in human retina. *J Comp Neurol* 1990; 300: 5–25.

Czeisler CA, Shanahan TL, Klerman EB, Martens H, Brotman DJ, Emens JS, et al. Suppression of melatonin secretion in some blind patients by exposure to bright light. *New England Journal of Medicine* 1995; 332: 6–11.

Daan S, Beersma DG, Borbély AA. Timing of human sleep: recovery process gated by a circadian pacemaker. *Am J Physiol* 1984; 246: R161-183.

Dacey DM, Liao HW, Peterson BB, Robinson FR, Smith VC, Pokorny J, et al. Melanopsin-expressing ganglion cells in primate retina signal colour and irradiance and project to the LGN. *Nature* 2005; 433: 749-754.

Davies WL, Hankins MW, Foster RG. Vertebrate ancient opsin and melanopsin: divergent irradiance detectors. *Photochem Photobiol Sci* 2010; 9:1444-1457.

Diederich NJ, Raman R, Leurgans S, Goetz CG. Progressive worsening of spatial and chromatic processing deficits in Parkinson disease. *Arch Neurol* 2002; 59: 1249-1252.

Diederich NJ, Vaillant M, Mancuso G, Lyen P, Tiete J. Progressive sleep 'destructuring' in Parkinson's disease. A polysomnographic study in 46 patients. *Sleep Med* 2005; 6: 313-318.

Diederich NJ, Pieri V, Hipp G, Rufra O, Blyth S, Vaillant M. Discriminative power of different non motor signs in early Parkinson's disease. A case-control study. *Mov Disord* 2010; 25:882-887.

Dijk DJ, Duffy JF, Czeisler CA. Contribution of circadian physiology and sleep homeostasis to age-related changes in human sleep. *Chronobiology International* 2000;17: 285–311.

Dijk DJ, Archer SN. Light, sleep, and circadian rhythms: together again. *PLoS Biol* 2009; 7: e1000145.

Dijk DJ, Archer SN. PERIOD3, circadian phenotypes, and sleep homeostasis. *Sleep Med Rev* 2010;14:151-160.

Do MT, Yau KW. Intrinsically photosensitive retinal ganglion cells. *Physiol Rev* 2010;90:1547-1581.

Dolman CL, McCormick AQ, Drance SM. Aging of the optic nerve. *Arch Ophthalmol* 1980;98:2053-8.

Dowling GA, Burr RL, Van Someren EJ, Hubbard EM, Luxenberg JS, Mastick J, Cooper BA. Melatonin and bright-light treatment for rest-activity disruption in institutionalized patients with Alzheimer's disease. *J Am Geriatr Soc* 2008;56:239-46.

Drouyer E, Dkhissi-Benyahya O, Chiquet C, WoldeMussie E, Ruiz G, Wheeler LA, et al. Glaucoma alters the circadian timing system. *PLoS One* 2008;e3931.

Duffy JF, Rimmer DW, Czeisler CA. Association of intrinsic circadian period with morningness-eveningness, usual wake time, and circadian phase. *Behav Neurosci* 2001;115:895-9.

Dutescu RM, Li QX, Crowston J, Masters CL, Baird PN, Culvenor JG. Amyloid precursor protein processing and retinal pathology in mouse models of Alzheimer'sdisease. *Graefes Arch Clin Exp Ophthalmol* 2009; 247:1213-1221.

Ecker JL, Dumitrescu ON, Wong KY, Alam NM, Chen SK, LeGates T, et al. Melanopsin-expressing retinal ganglion-cell photoreceptors: cellular diversity and role in pattern vision. *Neuron* 2010; 67: 49-60.

Ellis J, von Schantz M, Jones KH, Archer SN. Association between specific diurnal preference questionnaire items and PER3 VNTR genotype. *Chronobiol Int* 2009;26:464-73.

Esquiva G, González-Menédez G, Fernández-Sánchez L, García-Martín E, Pinilla I, García-Fernández J and Cuenca N. Degeneration of Melanopsin Photosensitive Ganglion Cells in Human Retinas With Aging and in Animal Models of Retinitis Pigmentosa. *Invest Ophthalmol Vis Sci* 2010;51: E-Abstract 680.

Feuer WJ, Budenz DL, Anderson DR, Cantor L, Greenfield DS, Savell J, et al. Topographic differences in the age-related changes in the retinal nerve fiber layer of normal eyes measured by Stratus optical coherence tomography. *J Glaucoma* 2011;20:133-8.

Fischer P, Jungwirth S, Zehetmayer S, Weissgram S, Hoenigschnabl S, Gelpi E, Krampla W, Tragl KH. Conversion from subtypes of mild cognitive impairment to Alzheimer dementia. *Neurology* 2007;68:288-91.

Folstein MF, Folstein SE, McHugh PR."Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *J Psychiatr Res* 1975;12:189-98.

Foster RG, Provencio I, Hudson D, Fiske S, De Grip W, Menaker M. Circadian photoreception in the retinally degenerate mouse (rd/rd*). Journal of Comparative Physiology A* 1991; 169: 39–50.

Foster RG. Neurobiology: bright blue times. *Nature* 2005; 433: 698-9.

Fotiou DF, Brozou CG, Haidich AB, Tsiptsios D, Nakou M, Kabitsi A, et al. Pupil reaction to light in Alzheimer's disease: evaluation of pupil size changes and mobility. *Aging Clin Exp Res* 2007; 19: 364-371.

Fotiou DF, Stergiou V, Tsiptsios D, Lithari C, Nakou M, Karlovasitou A. Cholinergic deficiency in Alzheimer's and Parkinson's disease: evaluation with pupillometry. *Int J Psychophysiol* 2009; 73: 143-149.

Franken P, Dijk DJ. Circadian clock genes and sleep homeostasis. *Eur J Neurosci* 2009; 29:1820-1829.

Freedman MS, Lucas RJ, Soni B, von Schantz M, Muñoz M, David-Gray Z, et al. Regulation of mammalian circadian behavior by non-rod, noncone, ocular photoreceptors. *Science* 1999; 284: 502–504.

Fronczek R, Overeem S, Lee SY, Hegeman IM, van Pelt J, van Duinen SG, et al. Hypocretin (orexin) loss in Parkinson's disease. *Brain* 2007; 130:1577-1585.

Gajula Balija MB, Griesinger C, Herzig A, Zweckstetter M, Jäckle H. Pre-fibrillar α-synuclein mutants cause Parkinson's disease-like non-motor symptoms in Drosophila. *PLoS One* 2011;6:e24701.

Gallassi R, Lenzi P, Stracciari A, Lorusso S, Ciardulli C, Morreale A, Mussuto V. Neuropsychological assessment of mental deterioration: purpose of a brief battery and a probabilistic definition of "normality" and "non-normality". *Acta Psychiatr Scand* 1986; 74:62-7.

Gallassi R, Morreale A, Di Sarro R, Lorusso S. Value of clinical data and neuropsychological measures in probable Alzheimer's disease. *Arch Gerontol Geriatr* 2002; 34:123-34.

Gelb DJ, Oliver E, Gilman S. Diagnostic criteria for Parkinson disease. *Arch Neurol* 1999;56:33-9.

Ghelli A, Porcelli AM, Zanna C, Martinuzzi A, Carelli V, Rugolo M. Protection against oxidant-induced apoptosis by exogenous glutathione in Leber hereditary optic neuropathy cybrids. *Invest Ophthalmol Vis Sci* 2008;49:671-6.

Giménez-Llort L, Blázquez G, Cañete T, Johansson B, Oddo S, Tobeña A, et al. Modeling behavioral and neuronal symptoms of Alzheimer's disease in mice: a role for intraneuronal amyloid. *Neurosci Biobehav Rev* 2007; 31:125-47.

Glickman G, Byrne B, Pineda C, Hauck WW, Brainard GC. Light therapy for seasonal affective disorder with blue narrow-band light-emitting diodes (LEDs). *Biological Psychiatry* 2006; 59: 502–507.

Golombek DA, Rosenstein RE. Physiology of circadian entrainment. *Physiol Rev* 2010;90:1063-102.

González-Menéndez I, Contreras F, Cernuda-Cernuda R, García-Fernández JM. No loss of melanopsin-expressing ganglion cells detected during postnatal development of the mouse retina. *Histol Histopathol* 2010; 25: 73-782.

Gooley JJ, Lu J, Chou TC, Scammell TE, Saper CB. Melanopsin in cells of origin of the retinohypothalamic tract. *Nat Neurosci* 2001; 4:1165.

Gravotta L, Gavrila AM, Hood S, Amir S. Global depletion of dopamine using intracerebroventricular 6-hydroxydopamine injection disrupts normal circadian wheel-running patterns and PERIOD2 expression in the rat forebrain. *J Mol Neurosci* 2011;45:162-71.

Guo L, Duggan J, Cordeiro MF. Alzheimer's disease and retinal neurodegeneration. *Curr Alzheimer Res* 2010;7:3-14.

Hajee ME, March WF, Lazzaro DR, Wolintz AH, Shrier EM, Glazman S, Bodis-Wollner IG. Inner retinal layer thinning in Parkinson disease. *Arch Ophthalmol* 2009; 127: 737-741.

Hannibal J, Vrang N, Card JP, Fahrenkrug J. Light-dependent induction of cFos during subjective day and night in PACAP-containing ganglion cells of the retinohypothalamic tract. *Journal of Biological Rhythms* 2001; 16: 457–470.

Hannibal J. Neurotransmitters of the retino-hypothalamic tract. *Cell Tissue Res* 2002a; 309: 73-88.

Hannibal J, Fahrenkrug J. Melanopsin: a novel photopigment involved in the photoentrainment of the brain's biological clock? *Ann Med* 2002b;34:401-7.

Hannibal J, Hindersson P, Ostergaard J, Georg B, Heegaard S, Larsen PJ, Fahrenkrug J. Melanopsin is expressed in PACAP-containing retinal ganglion cells of the human retinohypothalamic tract. *Invest Ophthalmol Vis Sci* 2004a; 45:4202-4209.

Hannibal J, Fahrenkrug J. Melanopsin containing retinal ganglion cells are light responsive from birth. *Neuroreport* 2004b; 15: 2317-2320.

Hannibal J, Fahrenkrug J. Neuronal input pathways to the brain's biological clock and their functional significance. *Adv Anat Embryol Cell Biol* 2006a; 182: 1-71.

Hannibal J. Roles of PACAP-containing retinal ganglion cells in circadian timing. *Int Rev Cytol* 2006b; 251: 1-39.

Hannibal J. Regulation of melanopsin expression. *Chronobiol Int* 2006c; 23:159-66.

Haq IZ, Naidu Y, Reddy P, Chaudhuri KR. Narcolepsy in Parkinson's disease. *Expert Rev Neurother* 2010;10:879-884.

Harand C, Bertran F, Doidy F, Guénolé F, Desgranges B, Eustache F, Rauchs G. How aging affects sleep-dependent memory consolidation? Front Neurol 2012;3:8.

Harnois C, Di Paolo T. Decreased dopamine in the retinas of patients with Parkinson's disease. *Invest Ophthalmol Vis Sci* 1990; 31:2473-2475.

Harper DG, Stopa EG, McKee AC, Satlin A, Harlan PC, Goldstein R, Volicer L. Differential circadian rhythm disturbances in men with Alzheimer disease and frontotemporal degeneration. *Arch Gen Psychiatry* 2001; 58:353-360

Harper DG, Volicer L, Stopa EG, McKee AC, Nitta M, Satlin A. Disturbance of endogenous circadian rhythm in aging and Alzheimer disease. *Am J Geriatr Psychiatry*. 2005;13:359-68.

Harper DG, Stopa EG, Kuo-Leblanc V, McKee AC, Asayama K, Volicer L, et al. Dorsomedial SCN neuronal subpopulations subserve different functions in human dementia. *Brain* 2008;131:1609-17.

Hatfield CF, Herbert J, van Someren EJ, Hodges JR, Hastings MH. Disrupted daily activity/rest cycles in relation to daily cortisol rhythms of home-dwelling patients with early Alzheimer's dementia. *Brain* 2004;127:1061-1074.

Hattar S, Liao HW, Takao M, Berson DM, Yau KW. Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. *Science* 2002; 295:1065-1070.

Hattar S, Kumar M, Park A, Tong P, Tung J, Yau KW, et al. Central projections of melanopsin-expressing retinal ganglion cells in the mouse. *J Comp Neurol* 2006; 497:326-349.

Heiduschka P, Julien S, Schuettauf F, Schnichels S. Loss of retinal function in aged DBA/2J mice - New insights into retinal neurodegeneration. *Exp Eye Res 2010*; 91:779-783.

Hinton DR, Sadun AA, Blanks JC, Miller CA. Optic-nerve degeneration inAlzheimer's disease. *N Engl J Med* 1986; 315:485-487.

Hof PR, Vogt BA, Bouras C, Morrison JH. Atypical form of Alzheimer's disease with prominent posterior cortical atrophy: a review of lesion distribution and circuit disconnection in cortical visual pathways. *Vision Res* 1997;37:3609-3625.

Hofman MA, Swaab DF. Alterations in circadian rhythmicity of the vasopressin-producing neurons of the human suprachiasmatic nucleus (SCN) with aging. *Brain Res* 1994;651:134-142.

Hofman MA. The human circadian clock and aging. *Chronobiol Int* 2000; 17:245-259.

Horne JA, Ostberg O. A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. *Int J Chronobiol*. 1976;4:97-110.

Hu K, Van Someren EJ, Shea SA, Scheer FA. Reduction of scale invariance of activity fluctuations with aging and Alzheimer's disease: Involvement of the circadian pacemaker. *Proc Natl Acad Sci U S A* 2009; 106:2490-2494.

Huang YL, Liu RY, Wang QS, Van Someren EJ, Xu H, Zhou JN. Age-associated difference in circadian sleep-wake and rest-activity rhythms. *Physiol Behav* 2002; 76:597-603.

Humbert W, Pévet P. Calcium content and concretions of pineal glands of young and old rats. A scanning and X-ray microanalytical study. *Cell Tissue Res* 1991; 263:593-6.

Hunt LA, Sadun AA, Bassi CJ. Review of the visual system in Parkinson's disease. *Optom Vis Sci* 1995; 72:92-99.

Hutton JT, Morris JL, Elias JW. Levodopa improves spatial contrast sensitivityin Parkinson's disease. *Arch Neurol* 1993; 50:721-724.

Hyman BT, Trojanowski JQ. Consensus recommendations for the postmortem diagnosis of Alzheimer disease from the National Institute on Aging and the Reagan Institute Working Group on diagnostic criteria for the neuropathological assessment of Alzheimer disease. *J Neuropathol Exp Neurol* 1997;56:1095-7.

Inzelberg R, Ramirez JA, Nisipeanu P, Ophir A. Retinal nerve fiber layer thinning in Parkinson disease. *Vision Res* 2004; 44:2793-2797.

Iranzo de Riquer A, Bergareche A, Campos V. Sleep disorders in Parkinson disease. *Neurologist* 2011;17:S38-42.

Iseri PK, Altinaş O, Tokay T, Yüksel N. Relationship between cognitive impairment and retinal morphological and visual functional abnormalities inAlzheimer disease. *J Neuroophthalmol* 2006; 26:18-24.

Isoldi MC, Rollag MD, Castrucci AM, Provencio I. Rhabdomeric phototransduction initiated by the vertebrate photopigment melanopsin. *Proc Natl Acad Sci U S A* 2005;102:1217-1221.

Jackson GR, Owsley C. Visual dysfunction, neurodegenerative diseases, and aging. *Neurol Clin* 2003; 21:7097-28.

Johnson BM, Miao M, Sadun AA. Age-related decline of human optic nerve axon populations. *Age* 1987; 10: 5–9.

Jones RD, Donaldson IM, Timmings PL. Impairment of high-contrast visual acuity in Parkinson's disease. *Mov Disord* 1992; 7:232-238.

Jusuf PR, Lee SC, Hannibal J, Grünert U. Characterization and synaptic connectivity of melanopsin-containing ganglion cells in the primate retina. *Eur J Neurosci* 2007; 26:2906-2921.

Kang JE, Lim MM, Bateman RJ, Lee JJ, Smyth LP, Cirrito JR, et al. Amyloid-beta dynamics are regulated by orexin and the sleep-wake cycle. *Science* 2009;326:1005-7.

Katz B, Rimmer S. Ophthalmologic manifestations of Alzheimer's disease. *Surv Ophthalmol* 1989a; 34:31-43.

Katz B, Rimmer S, Iragui V, Katzman R. Abnormal pattern electroretinogram in Alzheimer's disease: evidence for retinal ganglion cell degeneration? *Ann Neurol* 1989b; 26:221-225.

Kawasaki A, Kardon RH. Intrinsically photosensitive retinal ganglion cells. *J Neuroophthalmol* 2007; 27:195-204.

Kawasaki A, Herbst K, Sander B, Milea D. Selective wavelength pupillometry in Leber hereditary optic neuropathy. *Clinical and Experimental Ophthalmology* 2010; 38: 322–324.

Kesler A, Vakhapova V, Korczyn AD, Naftaliev E, Neudorfer M. Retinal thickness in patients with mild cognitive impairment and Alzheimer's disease. *Clin Neurol Neurosurg* 2011;113:523-6.

KJER P. Infantile optic atrophy with dominant mode of inheritance: a clinical and genetic study of 19 Danish families. *Acta Ophthalmol Suppl* 1959; 164:1-147.

Kjer P, Jensen OA, Klinken L. Histopathology of eye, optic nerve and brain in a case of dominant optic atrophy. *Acta Ophthalmol* 1983; 61:300–12.

Koronyo-Hamaoui M, Koronyo Y, Ljubimov AV, Miller CA, Ko MK, Black KL, et al. Identification of amyloid plaques in retinas from Alzheimer's patients and noninvasive in vivo optical imaging of retinal plaques in a mouse model. *Neuroimage* 2011;54:S204-17.

Koyanagi M, Terakita A. Gq-coupled rhodopsin subfamily composed of invertebrate visual pigment and melanopsin. *Photochem Photobiol* 2008; 84:1024-1030.

Krasodomska K, Lubiński W, Potemkowski A, Honczarenko K. Pattern electroretinogram (PERG) and pattern visual evoked potential (PVEP) in the early stages of Alzheimer's disease. *Doc Ophthalmol* 2010; 121:111-121.

Kudo T, Loh DH, Truong D, Wu Y, Colwell CS. Circadian dysfunction in a mouse model of Parkinson's disease. *Exp Neurol* 2011;232:66-75.

La Morgia C, Ross-Cisneros FN, Sadun AA, Hannibal J, Munarini A, Mantovani V, et al. Melanopsin retinal ganglion cells are resistant to neurodegeneration in mitochondrial optic neuropathies. *Brain* 2010; 133:2426-2438.

La Morgia C, Ross-Cisneros FN, Hannibal J, Montagna P, Sadun AA, Carelli V. Melanopsin-expressing retinal ganglion cells: implications for human diseases. *Vision Res* 2011; 51:296-302.

La Morgia C, Ross-Cisneros FN, Hannibal J, Sadun AA, Carelli V, Montagna P. Sleep and circadian rhythms in neurodegenerative disorders: role of melanopsin expressing retinal ganglion cells. Luka Golovkin and Alexei Maliszkewicz, editors. Circadian Rhythms: Biology, Cognition and Disorders. Nova Publishers, Inc.; 2012a. Chapter 13, p. 293-326.

La Morgia C, Barboni P, Rizzo G, Carbonelli M, Savini G, Scaglione C, et al. Loss of temporal retinal nerve fibers in Parkinson disease: a mitochondrial pattern? *Eur J Neurol*. 2012b Mar 21. doi:10.1111/j.1468-1331.2012.03701.x. [Epub ahead of print]

Lall GS, Revell VL, Momiji H, Al Enezi J, Altimus CM, Güler AD, et al. Distinct contributions of rod, cone, and melanopsin photoreceptors to encoding irradiance. *Neuron* 2010; 66:417-428.

Langston JW, Forno LS. The hypothalamus in Parkinson disease. *Ann Neurol* 1978;3:129-133.

Lax P, Otalora BB, Esquiva G, Rol Mde L, Madrid JA, Cuenca N. Circadian dysfunction in P23H rhodopsin transgenic rats: effects of exogenous melatonin. *J Pineal Res* 2011;50:183-91.

Lee HJ, Rex KM, Nievergelt CM, Kelsoe JR, Kripke DF. Delayed sleep phase syndrome is related to seasonal affective disorder. *J Affect Disord* 2011;133:573-9.

Lewy AJ, Lefler BJ, Emens JS, Bauer VK. The circadian basis of winter depression. *Proc Natl Acad Sci U S A* 2006;103:7414-9.

Lewy AJ. Circadian misalignment in mood disturbances. *Current Psychiatry Reports* 2009a; 11 :459–465.

Lewy AJ, Emens JS, Songer JB, Sims N, Laurie AL, Fiala SC, et al. Winter depression: Integrating mood, circadian rhythms, and the sleep/wake and light/dark cycles into a bio-psycho-social–environmental model. *Sleep Medicine Clinics* 2009b;4: 285–299.

Li SY, Yau SY, Chen BY, Tay DK, Lee V, Pu ML, et al. Enhanced survival of melanopsin-expressing retinal ganglion cells after injury is associated with the PI3 K/Akt pathway. *Cellular and Molecular Neurobiology* 2008; 28: 1095–1107.

Liu RY, Zhou JN, van Heerikhuize J, Hofman MA, Swaab DF. Decreased melatonin levels in postmortem cerebrospinal fluid in relation to aging, Alzheimer's disease, and apolipoprotein E-epsilon4/4 genotype. *J Clin Endocrinol Metab* 1999 ; 84:323-327.

Liu B, Rasool S, Yang Z, Glabe CG, Schreiber SS, Ge J, Tan Z. Amyloid-peptide vaccinations reduce {beta}-amyloid plaques but exacerbate vascular deposition and inflammation in the retina of Alzheimer's transgenic mice. *Am J Pathol* 2009; 175:2099-2110.

Löffler KU, Edward DP, Tso MO. Immunoreactivity against tau, amyloid precursor protein, and beta-amyloid in the human retina. *Invest Ophthalmol Vis Sci* 1995; 36:24-31.

Lu Y, Li Z, Zhang X, Ming B, Jia J, Wang R, Ma D. Retinal nerve fiber layer structure abnormalities in early Alzheimer's disease: evidence in optical coherence tomography. *Neurosci Lett* 2010; 480:69-72.

Lucas RJ, Freedman MS, Muñoz M, Garcia-Fernández JM, Foster RG. Regulation of the mammalian pineal by non-rod, non-cone, ocular photoreceptors. *Science* 1999, 284: 505–507.

Lupi D, Oster H, Thompson S, Foster RG. The acute light-induction of sleep is mediated by OPN4-based photoreception. *Nat Neurosci* 2008; 11:1068-1073

Lupi D, Semo M, Foster RG. Impact of age and retinal degeneration on the light input to circadian brain structures. *Neurobiol Aging* 2012;33:383-92.

Magnusson A. Validation of the Seasonal Pattern Assessment Questionnaire (SPAQ). *J Affect Disord* 1996;40:121-9.

Matsui H, Udaka F, Tamura A, Oda M, Kubori T, Nishinaka K, et al. Impaired visual acuity as a risk factor for visual hallucinations in Parkinson's disease. *J Geriatr Psychiatry Neurol* 2006;19:36-40.

McCurry SM, Logsdon RG, Teri L, Gibbons LE, Kukull WA, Bowen JD, et al. Characteristics of sleep disturbance in community-dwelling Alzheimer's disease patients. *J Geriatr Psychiatry Neurol* 1999;12:53-9.

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* 1984;34:939-44.

Mendez MF, Mendez MA, Martin R, Smyth KA, Whitehouse PJ. Complex visual disturbances in Alzheimer's disease. *Neurology* 1990; 40:439-443.

Menza M, Dobkin RD, Marin H, Bienfait K. Sleep disturbances in Parkinson's disease. *Mov Disord* 2010;25:S117-22.

Meppelink AM, de Jong BM, Renken R, Leenders KL, Cornelissen FW, van Laar T. Impaired visual processing preceding image recognition in Parkinson's disease patients with visual hallucinations. *Brain* 2009; 132:2980-2993.

Mirmiran M, Swaab DF, Kok JH, Hofman MA, Witting W, Van Gool WA. Circadian rhythms and the suprachiasmatic nucleus in perinatal development, aging and Alzheimer's disease. *Prog Brain Res* 1992;93:151-62.

Mirra, SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, et al. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD) Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology* 1991;41:479–486.

Mishima K, Okawa M, Satoh K, Shimizu T, Hozumi S, Hishikawa Y. Different manifestations of circadian rhythms in senile dementia of Alzheimer's type and multi-infarct dementia. *Neurobiol Aging* 1997;18:105-9.

Mishima K, Tozawa T, Satoh K, Matsumoto Y, Hishikawa Y, Okawa M. Melatonin secretion rhythm disorders in patients with senile dementia of Alzheimer's type with disturbed sleep-waking. *Biol Psychiatry* 1999;45:417-21.

Mistlberger RE. Circadian regulation of sleep in mammals: role of the suprachiasmatic nucleus. *Brain Res Brain Res Rev* 2005; 49:429-454.

Moe KE, Vitiello MV, Larsen LH, Prinz PN. Symposium: Cognitive processes and sleep disturbances: Sleep/wake patterns in Alzheimer's disease: relationship with cognition and function. *J Sleep Res* 1995;4:15-20.

Mongrain V, Carrier J, Dumont M. Circadian and homeostatic sleep regulation in morningness-eveningness. *J Sleep Res* 2006; 15:162-166.

Mongrain V, Dumont M. Increased homeostatic response to behavioral sleep fragmentation in morning types compared to evening types. *Sleep* 2007; 30:773-780.

Montine TJ, Phelps CH, Beach TG, Bigio EH, Cairns NJ, Dickson DW, et al. National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease: a practical approach. *Acta Neuropathol* 2012;123:1-11.

Moore RY, Lenn NJ. A retinohypothalamic projection in the rat. *J Comp Neurol* 1972; 146:1-14.

Moore RY, Speh JC, Card JP. The retinohypothalamic tract originates from a distinct subset of retinal ganglion cells. *J Comp Neurol* 1995; 352:351-366.

Moran M, Lynch CA, Walsh C, Coen R, Coakley D, Lawlor BA. Sleep disturbance in mild to moderate Alzheimer's disease. *Sleep Med* 2005;6:347-52.

Moschos MM, Tagaris G, Markopoulos I, Margetis I, Tsapakis S, Kanakis M, Koutsandrea C. Morphologic changes and functional retinal impairment in patients with Parkinson disease without visual loss. *Eur J Ophthalmol* 2011;21:24-29.

Moura AL, Nagy BV, La Morgia C, Salomao SR, Berezovsky A, Chicani CF, et al. Melanopsin Driven Pupillary Light Response in Leber's Hereditary Optic Neuropathy (LHON). *Invest Ophthalmol Vis Sci* 2011;52: E-Abstract 5312.

Muscettola G, Barbato G, Ficca G, Beatrice M, Puca M, Aguglia E, et al. Seasonality of mood in Italy: role of latitude and socio- cultural factors. *Journal of Affective Disorder* 1995, 33, 135-139

Neikrug AB, Ancoli-Israel S. Sleep disorders in the older adult – a mini-review. *Gerontology* 2010;56: 181-189.

Netzer NC, Stoohs RA, Netzer CM, Clark K, Strohl KP. Using the Berlin Questionnaire to identify patients at risk for the sleep apnea syndrome. *Ann Intern Med* 1999;13:485-91.

Nguyen-Legros J. Functional neuroarchitecture of the retina: hypothesis on the dysfunction of retinal dopaminergic circuitry in Parkinson's disease. *Surg Radiol Anat* 1988; 10:137-144.

Ning A, Cui J, To E, Ashe KH, Matsubara J. Amyloid-beta deposits lead to retinal degeneration in a mouse model of Alzheimer disease. *Invest Ophthalmol VisSci* 2008;49: 5136-5143.

Niwa F, Kuriyama N, Nakagawa M, Imanishi J. Circadian rhythm of rest activity and autonomic nervous system activity at different stages in Parkinson's disease. *Auton Neurosci* 2011;165:195-200.

Noseda R, Kainz V, Jakubowski M, Gooley JJ, Saper CB, Digre K, Burstein R. A neural mechanism for exacerbation of headache by light. *Nat Neurosci* 2010;13:239-45.

Noseda R, Burstein R. Advances in understanding the mechanisms of migraine-type photophobia. *Curr Opin Neurol* 2011;24:197-202.

Ohashi Y, Okamoto N, Uchida K, Iyo M, Mori N, Morita Y. Daily rhythm of serum melatonin levels and effect of light exposure in patients with dementia of the Alzheimer's type. *Biol Psychiatry* 1999;45:1646-1652.

Okawa M, Mishima K, Hishikawa Y, Hozumi S, Hori H, Takahashi K. Circadian rhythm disorders in sleep-waking and body temperature in elderly patients with dementia and their treatment. *Sleep* 1991;14:478-485.

Oosterman JM, van Someren EJ, Vogels RL, Van Harten B, Scherder EJ. Fragmentation of the rest-activity rhythm correlates with age-related cognitive deficits. *J Sleep Res* 2009; 18:129-135.

Osborne NN, Li GY, Ji D, Mortiboys HJ, Jackson S. Light affects mitochondria to cause apoptosis to cultured cells: possible relevance to ganglion cell death in certain optic neuropathies. *J Neurochem* 2008;105: 2013–28.

Otsuka A, Mikami H, Katahira K, Nakamoto Y, Minamitani K, Imaoka M, et al. Absence of nocturnal fall in blood pressure in elderly persons with Alzheimer-type dementia. *J Am Geriatr Soc* 1990;38:973-8.

Pace-Schott EF, Spencer RM. Age-related changes in the cognitive function of sleep. *Prog Brain Res* 2011;191:75-89.

Paquet C, Boissonnot M, Roger F, Dighiero P, Gil R, Hugon J. Abnormal retinal thickness in patients with mild cognitive impairment and Alzheimer's disease. *Neurosci Lett* 2007; 420:97-99.

Park JC, Moura AL, Raza AS, Rhee DW, Kardon RH, Hood DC. Toward a clinical protocol for assessing rod, cone, and melanopsin contributions to the human pupil response. *Invest Ophthalmol Vis Sci* 2011;52:6624-35.

Parisi V, Restuccia R, Fattapposta F, Mina C, Bucci MG, Pierelli F. Morphological and functional retinal impairment in Alzheimer's disease patients. *Clin Neurophysiol* 2001;112: 1860-1867.

Parisi V. Correlation between morphological and functional retinal impairment in patients affected by ocular hypertension, glaucoma, demyelinating optic neuritis and Alzheimer's disease. *Semin Ophthalmol* 2003; 18:50-57.

Paus S, Schmitz-Hübsch T, Wüllner U, Vogel A, Klockgether T, Abele M. Bright light therapy in Parkinson's disease: a pilot study. *Mov Disord* 2007;22:1495-1498.

Peirson SN, Thompson S, Hankins MW, Foster RG. Mammalian photoentrainment: results, methods, and approaches. *Methods Enzymol* 2005;393:697-726.

Peppe A, Stanzione P, Pierelli F, De Angelis D, Pierantozzi M, Bernardi G.Visual alterations in de novo Parkinson's disease: pattern electroretinogram latencies are more delayed and more reversible by levodopa than are visual evoked potentials. *Neurology* 1995;45:1144-1148.

Peppe A, Stanzione P, Pierantozzi M, Semprini R, Bassi A, Santilli AM, et al. Does pattern electroretinogram spatial tuning alteration in Parkinson's disease depend on motor disturbances or retinal dopaminergic loss? *Electroencephalogr Clin Neurophysiol* 1998; 106:374-382.

Perez SE, Lumayag S, Kovacs B, Mufson EJ, Xu S. Beta-amyloid deposition and functional impairment in the retina of the APPswe/PS1DeltaE9 transgenic mouse model of Alzheimer's disease. *Invest Ophthalmol Vis Sci* 2009; 50:793-800.

Pérez-Rico C, de la Villa P, Blanco R, Germain F, Paz-Moreno J, Arribas-Gómez I. [Alterations in nocturnal melatonin secretion in patients with opticneuropathies]. *Arch Soc Esp Oftalmol* 2009;84:251-257.

Pérez-Rico C, de la Villa P, Arribas-Gómez I, Blanco R. Evaluation of functional integrity of the retinohypothalamic tract in advanced glaucoma using multifocal electroretinography and light-induced melatonin suppression. *Exp Eye Res* 2010;91:578-83.

Perrin F, Peigneux P, Fuchs S, Verhaeghe S, Laureys S, Middleton B, et al. Nonvisual responses to light exposure in the human brain during the circadian night. *Curr Biol*. 2004;14:1842-6.

Petersen RC, Doody R, Kurz A, Mohs RC, Morris JC, Rabins PV, et al. Current concepts in mild cognitive impairment. *Arch Neurol* 2001;58:1985-92.

Pfeffer M, Plenzig S, Gispert S, Wada K, Korf HW, Von Gall C. Disturbed sleep/wake rhythms and neuronal cell loss in lateral hypothalamus and retina of mice with a spontaneous deletion in the ubiquitin carboxyl-terminal hydrolase L1 gene. *Neurobiol Aging* 2012;33:393-403.

Poewe W, Högl B. Parkinson's disease and sleep. *Curr Opin Neurol* 2000; 13:423-426.

Pollock VE, Schneider LS, Chui HC, Henderson V, Zemansky M, Sloane RB. Visual evoked potentials in dementia: a meta-analysis and empirical study of Alzheimer'sdisease patients. *Biol Psychiatry* 1989; 25:1003-1013.

Porcelli AM, Ghelli A, Iommarini L, Mariani E, Hoque M, et al. The antioxidant function of Bcl-2 preserves cytoskeletal stability of cells with defective respiratory complex I. *Cell Mol Life Sci* 2008; 65: 2943-2951

Postuma RB, Gagnon JF, Vendette M, Charland K, Montplaisir J. Manifestations of Parkinson disease differ in association with REM sleep behavior disorder. *Mov Disord* 2008; 23:1665-1672.

Postuma RB, Gagnon JF, Vendette M, Montplaisir JY. Markers of neurodegeneration in idiopathic rapid eye movement sleep behaviour disorder and Parkinson's disease. *Brain* 2009; 132:3298-3307.

Price MJ, Feldman RG, Adelberg D, Kayne H. Abnormalities in color vision and contrast sensitivity in Parkinson's disease. *Neurology* 1992;42:887-890.

Prinz PN, Peskind ER, Vitaliano PP, Raskind MA, Eisdorfer C, Zemcuznikov N, et al. Changes in the sleep and waking EEGs of non demented and demented elderly subjects. *J Am Geriatr Soc* 1982; 30:86-93.

Provencio I, Jiang G, De Grip WJ, Hayes WP, Rollag MD. Melanopsin: An opsin in melanophores, brain, and eye. *Proc Natl Acad Sci U S A* 1998; 95:340-345.

Provencio I, Rodriguez IR, Jiang G, Hayes WP, Moreira EF, Rollag MD. A novel human opsin in the inner retina. *J Neurosci* 2000; 20:600-605.

Provencio I, Rollag MD, Castrucci AM. Photoreceptive net in the mammalian retina. This mesh of cells may explain how some blind mice can still tell day from night. *Nature* 2002; 415:493.

Pursiainen V, Haapaniemi TH, Korpelainen JT, Huikuri HV, Sotaniemi KA, Myllylä VV. Circadian heart rate variability in Parkinson's disease. *J Neurol* 2002; 249:1535-1540.

Regan D, Neima D. Low-contrast letter charts in early diabetic retinopathy, ocular hypertension, glaucoma, and Parkinson's disease. *Br J Ophthalmol* 1984; 68:885-889.

Reynolds CF 3rd, Hoch CC, Stack J, Campbell D. The nature and management of sleep/wake disturbance in Alzheimer's dementia. *Psychopharmacol Bull* 1988; 24:43-8.

Reynolds V, Marriott FH, Waterhouse J et al. Heart rate variation, age, and behavior in subjects with senile dementia of Alzheimer type. *Chronobiol Int* 1995;12: 37–45.

Riemersma-van der Lek RF, Swaab DF, Twisk J, Hol EM, Hoogendijk WJ, Van Someren EJ. Effect of bright light and melatonin on cognitive and noncognitive function in elderly residents of group care facilities: a randomized controlled trial. *JAMA* 2008;299:2642-55.

Robinson GA, Madison RD. Axotomized mouse retinal ganglion cells containing melanopsin show enhanced survival, but not enhanced axonregrowth into a peripheral nerve graft. *Vision Research* 2004; 44: 2667–2674.

Roecklein KA, Rohan KJ, Duncan WC, Rollag MD, Rosenthal NE, Lipsky RH, et al. A missense variant (P10L) of the melanopsin (OPN4) gene in seasonal affective disorder. *J Affect Disord* 2009; 114:279-285.

Rosenthal NE, Sack DA, Gillin DC, Lewy AJ, Goodwin FK, Davenport Y, et al. Seasonal affective disorder. A description of the syndrome and preliminary findings with light therapy. *Archives of General Psychiatry* 1984, 41, 72-80.

Rosenwasser AM. Circadian clock genes: non-circadian roles in sleep, addiction, and psychiatric disorders? *Neurosci Biobehav Rev* 2010; 34:1249-1255.

Sadun AA, Schaechter JD, Smith LE. A retinohypothalamic pathway in man: light mediation of circadian rhythms. *Brain Res* 1984; 302:371-377.

Sadun AA, Borchert M, DeVita E, Hinton DR, Bassi CJ. Assessment of visual impairment in patients with Alzheimer's disease. *Am J Ophthalmol* 1987; 104:113-120.

Sadun AA, Bassi CJ. Optic nerve damage in Alzheimer's disease. *Ophthalmology* 1990; 97:9-17.

Sadun AA, Carelli V, Salomao SR, Berezovsky A, Quiros PA, Sadun F, et al. Extensive investigation of a large Brazilian pedigree of 11778/ haplogroup J Leber hereditary optic neuropathy. *Am J Ophthalmol* 2003; 136: 231–8.

Sakamoto K, Liu C, Kasamatsu M, Pozdeyev NV, Iuvone PM, Tosini G. Dopamine regulates melanopsin mRNA expression in intrinsically photosensitive retinalganglion cells. *Eur J Neurosci* 2005; 22:3129-3136.

Sánchez-Hidalgo M, Guerrero Montávez JM, Carrascosa-Salmoral Mdel P, Naranjo Gutierrez Mdel C, Lardone PJ, de la Lastra Romero CA. Decreased MT1 and MT2 melatonin receptor expression in extrapineal tissues of the rat during physiological aging. *J Pineal Res* 2009; 46:29-35.

Saper CB, Lu J, Chou TC, Gooley J. The hypothalamic integrator for circadian rhythms. *Trends Neurosci* 2005a; 28:152-157.

Saper CB, Scammell TE, Lu J. Hypothalamic regulation of sleep and circadian rhythms. *Nature* 2005b;437:1257-1263.

Sartucci F, Borghetti D, Bocci T, Murri L, Orsini P, Porciatti V, et al. Dysfunction of the magnocellular stream in Alzheimer's disease evaluated by pattern electroretinograms and visual evoked potentials. *Brain Res Bull* 2010;82:169-176

Satlin A, Volicer L, Stopa EG, Harper D. Circadian locomotor activity and core-body temperature rhythms in Alzheimer's disease. *Neurobiol Aging* 1995;16:765-771.

Schapira AH. Mitochondria in the aetiology and pathogenesis of Parkinson's disease. *Lancet Neurol* 2008:97-109.

Schmidt, TM, Kofuji P. Functional and morphological differences among intrinsically photosensitive retinal ganglion cells. *J Neurosci* 2009; 29: 476–482.

Schmidt TM, Kofuji P. Differential cone pathway influence on intrinsically photosensitive retinal ganglion cell subtypes. *J Neurosci* 2010; 30:16262-16271.

Schmidt TM, Do MT, Dacey D, Lucas R, Hattar S, Matynia A. Melanopsin-positive intrinsically photosensitive retinal ganglion cells: from form to function. *J Neurosci* 2011a;31:16094-101.

Schmidt TM, Chen SK, Hattar S. Intrinsically photosensitive retinal ganglion cells: many subtypes, diverse functions. *Trends Neurosci* 2011b;34:572-80.

Schmidt C, Peigneux P, Leclercq Y, Sterpenich V, Vandewalle G, Phillips C, et al. Circadian preference modulates the neural substrate of conflict processing across the day. PLoS One 2012;7:e29658.

Sekaran S, Lupi D, Jones SL, Sheely CJ, Hattar S, Yau KW, et al. Melanopsin-dependent photoreception provides earliest light detection in the mammalian retina. *Curr Biol* 2005;15:1099-1107.

Semo M, Peirson S, Lupi D, Lucas RJ, Jeffery G, Foster RG. Melanopsin retinal ganglion cells and the maintenance of circadian and pupillary responses to light in aged rodless/coneless (rd/rd cl) mice. *Eur J Neurosci* 2003;17:1793-1801.

Senard JM, Chamontin B, Rascol A, Montastruc JL. Ambulatory blood pressure in patients with Parkinson's disease without and with orthostatic hypotension. *Clin Auton Res* 1992; 2:99-104.

Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM, Sirotkin K. dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res* 2001; 29: 308–311.

Silva MF, Faria P, Regateiro FS, Forjaz V, Januário C, Freire A, et al. Independent patterns of damage within magno-, parvo- and koniocellular pathways in Parkinson's disease. *Brain* 2005; 128:2260-2271.

Simuni T, Sethi K. Nonmotor manifestations of Parkinson's disease. *Ann Neurol* 2008;64 Suppl 2:S65-80.

Skene DJ, Vivien-Roels B, Sparks DL, Hunsaker JC, Pévet P, Ravid D, et al. Daily variation in the concentration of melatonin and 5-methoxytryptophol in the human pineal gland: effect of age and Alzheimer's disease. *Brain Res* 1990; 528:170-174.

Stavitsky K, Saurman JL, McNamara P, Cronin-Golomb A. Sleep in Parkinson's disease: a comparison of actigraphy and subjective measures. *Parkinsonism Relat Disord* 2010;16:280-3.

Stavitsky K, Neargarder S, Bogdanova Y, McNamara P, Cronin-Golomb A. The impact of sleep quality on cognitive functioning in Parkinson's disease. *J Int Neuropsychol Soc* 2012;18:108-17.

Steinmeyer C, Mueller JC, Kempenaers B. Search for informative polymorphisms in candidate genes: clock genes and circadian behaviour in blue tits. *Genetica* 2009;136:109-17.

Sterniczuk R, Dyck RH, Laferla FM, Antle MC. Characterization of the 3xTg-AD mouse model of Alzheimer's disease: part 1. Circadian changes. *Brain Res* 2010; 1348:139-148.

Stopa EG, Volicer L, Kuo-Leblanc V, Harper D, Lathi D, Tate B, Satlin A. Pathologic evaluation of the human suprachiasmatic nucleus in severe dementia. *J Neuropathol Exp Neurol* 1999; 58:29-39.

Storoni M, Plant GT, Bi W, Barbur JL. Selective Sparing Of Rod / Melanopsin Driven Ganglion Cells In Leber's Hereditary Optic Neuropathy (LHON). *Invest Ophthalmol Vis Sci* 2011;52: E-Abstract 5313.

Swaab DF, Fliers E, Partiman TS. The suprachiasmatic nucleus of the human brain in relation to sex, age and senile dementia. *Brain Res* 1985; 342:37-44.

Swaab DF, Roozendaal B, Ravid R, Velis DN, Gooren L, Williams RS. Suprachiasmatic nucleus in aging, Alzheimer's disease, transsexuality and Prader-Willi syndrome. *Prog Brain Res* 1987; 72:301-310.

Tagliati M, Bodis-Wollner I, Yahr MD. The pattern electroretinogram in Parkinson's disease reveals lack of retinal spatial tuning. *Electroencephalogr Clin Neurophysiol* 1996; 100:1-11.

Tamura H, Kawakami H, Kanamoto T, Kato T, Yokoyama T, Sasaki K, et al. High frequency of open-angle glaucoma in Japanese patients with Alzheimer's disease. *J Neurol Sci* 2006;246:79-83.

Tenhula WN, Xu SZ, Madigan MC, Heller K, Freeman WR, Sadun AA. Morphometric comparisons of optic nerve axon loss in acquired immunodeficiency syndrome. *Am J Ophthalmol*. 1992;113:14-20.

Thapan K, Arendt J, Skene DJ. An action spectrum for melatonin suppression: evidence for a novel non-rod, non-cone photoreceptor system in humans. *J Physiol* 2001; 535:261-267.

Thiselton DL, Alexander C, Taanman JW, Brooks S, Rosenberg T, Eiberg H, et al. A comprehensive survey of mutations in the OPA1 gene in patients with autosomal dominant optic atrophy. *Invest Ophthalmol Vis Sci* 2002; 43: 1715–24.

Thorpy MJ, Adler CH. Parkinson's disease and sleep. *Neurol Clin* 2005; 23:1187-1208.

Tobler I, Deboer T. Sleep in the blind mole rat Spalax ehrenbergi. *Sleep* 2001; 24:147-154.

Touitou Y, Reinberg A, Bogdan A, Auzéby A, Beck H, Touitou C. Age-related changes in both circadian and seasonal rhythms of rectal temperature with special reference to senile dementia of Alzheimer type. *Gerontology* 1986; 32:110-118.

Tractenberg RE, Singer CM, Kaye JA. Characterizing sleep problems in persons with Alzheimer's disease and normal elderly. *J Sleep Res*. 2006;15:97-103.

Tranah GJ, Blackwell T, Stone KL, Ancoli-Israel S, Paudel ML, Ensrud KE, Cauley JA, Redline S, Hillier TA, Cummings SR, Yaffe K; SOF Research Group. Circadian activity rhythms and risk of incident dementia and mild cognitive impairment in older women. *Ann Neurol* 2011;70:722-32.

Trick GL, Barris MC, Bickler-Bluth M. Abnormal pattern electroretinograms in patients with senile dementia of the Alzheimer type. *Ann Neurol* 1989; 26:226-231.

Tsai JW, Hannibal J, Hagiwara G, Colas D, Ruppert E, Ruby NF, et al. Melanopsin as a sleep modulator: circadian gating of the direct effects of light on sleep and altered sleep homeostasis in Opn4(-/-) mice. *PLoS Biol* 2009; 7:e1000125.

Tu DC, Zhang D, Demas J, Slutsky EB, Provencio I, Holy TE, Van Gelder RN. Physiologic diversity and development of intrinsically photosensitive retinal ganglion cells. *Neuron* 2005; 48:987-999.

Turner PL, Mainster MA. Circadian photoreception: ageing and the eye's important role in systemic health. *Br J Ophthalmol* 2008; 92:1439-1444.

Uchida K, Okamoto N, Ohara K, Morita Y. Daily rhythm of serum melatonin in patients with dementia of the degenerate type. *Brain Res* 1996; 717:154-159.

van Hilten B, Hoff JI, Middelkoop HA, van der Velde EA, Kerkhof GA, Wauquier A, Kamphuisen HA, Roos RA. Sleep disruption in Parkinson's disease. Assessment by continuous activity monitoring. *Arch Neurol* 1994;51:922-8.

Van Someren EJ, Hagebeuk EE, Lijzenga C, Scheltens P, de Rooij SE, Jonker C, Pot AM, Mirmiran M, Swaab DF. Circadian rest-activity rhythm disturbances in Alzheimer's disease. *Biol Psychiatry* 1996 15; 40:259-720.

Van Someren EJ. Actigraphic monitoring of movement and rest-activity rhythms in aging, Alzheimer's disease, and Parkinson's disease. *IEEE Trans Rehabil Eng* 1997a; 5:394-398.

Van Someren EJ, Kessler A, Mirmiran M, Swaab DF. Indirect bright light improves circadian rest-activity rhythm disturbances in demented patients. *Biol Psychiatry*. 1997b;41:955-63.

Van Someren EJ, Swaab DF, Colenda CC, Cohen W, McCall WV, Rosenquist PB. Bright light therapy: improved sensitivity to its effects on rest-activity rhythms in Alzheimer patients by application of nonparametric methods. *Chronobiol Int* 1999;16:505-18.

Van Someren EJ. Circadian rhythms and sleep in human aging. *Chronobiol Int 2000*; 17:233-243.

Van Someren EJ. Improving actigraphic sleep estimates in insomnia and dementia: how many nights? *J Sleep Res* 2007;16:269-75.

Vandewalle G, Schmidt C, Albouy G, Sterpenich V, Darsaud A, Rauchs G, et al. Brain responses to violet, blue, and green monochromatic light exposures in humans: prominent role of blue light and the brainstem. *PLoS One* 2007; 2:e1247.

Vandewalle G, Maquet P, Dijk DJ. Light as a modulator of cognitive brain function. *Trends Cogn Sci*. 2009a; 13:429-438.

Vandewalle G, Archer SN, Wuillaume C, Balteau E, Degueldre C, Luxen A, et al. Functional magnetic resonance imaging-assessed brain responses during an executive task depend on interaction of sleep homeostasis, circadian phase, and PER3 genotype. *J Neurosci*. 2009b;29:7948-56.

Vandewalle G, Schwartz S, Grandjean D, Wuillaume C, Balteau E, Degueldre C, et al. Spectral quality of light modulates emotional brain responses in humans. *Proc Natl Acad Sci U S A* 2010; 107:19549-19554.

Vandewalle G, Archer SN, Wuillaume C, Balteau E, Degueldre C, Luxen A, et al. Effects of light on cognitive brain responses depend on circadian phase and sleep homeostasis. *J Biol Rhythms* 2011a;26:249-59.

Vandewalle G, Hébert M, Beaulieu C, Richard L, Daneault V, Garon ML, et al. Abnormal hypothalamic response to light in seasonal affective disorder. *Biol Psychiatry* 2011b;70:954-61.

Vezoli J, Fifel K, Leviel V, Dehay C, Kennedy H, Cooper HM, et al. Early presymptomatic and long-term changes of rest activity cycles and cognitive behavior in a MPTP-monkey model of Parkinson's disease. *PLoS One* 2011;6:e23952.

Vignatelli L, Plazzi G, Barbato A, Ferini-Strambi L, Manni R, Pompei F, D'Alessandro R; GINSEN (Gruppo Italiano Narcolessia Studio Epidemiologico Nazionale. Italian version of the Epworth sleepiness scale: external validity. *Neurol Sci* 2003;23:295-300.

Viney TJ, Balint K, Hillier D, Siegert S, Boldogkoi Z, Enquist LW, et al. Local retinal circuits of melanopsin-containing ganglion cells identified by transsynaptic viral tracing. *Curr Biol* 2007;17:981-988.

Viola AU, Archer SN, James LM, Groeger JA, Lo JC, Skene DJ, et al. PER3 polymorphism predicts sleep structure and waking performance. *Curr Biol* 2007; 17:613-618.

Visser SL, Stam FC, van Tilburg W, den Velde W, Blom JL, de Rijke W. Visual evoked response in senile and presenile dementia. *Electroencephalogr Clin Neurophysiol* 1976; 40:385-392.

Visser SL, Van Tilburg W, Hooijer C, Jonker C, De Rijke W. Visual evoked potentials (VEPs) in senile dementia (Alzheimer type) and in non-organic behavioural disorders in the elderly; comparison with EEG parameters. *Electroencephalogr Clin Neurophysiol* 1985; 60:115-121.g

Vitiello MV, Prinz PN. Alzheimer's disease. Sleep and sleep/wake patterns. *Clin Geriatr Med* 1989;5:289-299.

Vitiello MV, Poceta JS, Prinz PN. Sleep in Alzheimer's disease and other dementing disorders. *Can J Psychol* 1991; 45:221-239.

Vives-Bauza C, Przedborski S. Mitophagy: the latest problem for Parkinson's disease. *Trends Mol Med* 2011;17:158-65.

Volicer L, Harper DG, Manning BC, Goldstein R, Satlin A. Sundowning and circadian rhythms in Alzheimer's disease. *Am J Psychiatry* 2001;158:704-711.

von Bussmann KA, Garey LJ, Jen LS. Injury-resistant retinal ganglion cells that are rich in cytochrome oxidase. *NeuroReport* 1993; 4: 247–250.

Vugler AA, Redgrave P, Semo M, Lawrence J, Greenwood J, Coffey PJ. Dopamine neurones form a discrete plexus with melanopsin cells in normal and degenerating retina. *Exp Neurol* 2007; 205:26-35.

Wenzelburger R, Zhang BR, Pohle S, Klebe S, Lorenz D, Herzog J, et al. Force overflow and levodopa-induced dyskinesias in Parkinson's disease. *Brain* 2002;125:871-9.

Westerberg CE, Lundgren EM, Florczak SM, Mesulam MM, Weintraub S, Zee PC, Paller KA. Sleep Influences the Severity of Memory Disruption in Amnestic Mild Cognitive Impairment: Results From Sleep Self-assessment and Continuous Activity Monitoring. *Alzheimer Dis Assoc Disord*. 2010;24:325–333

White KE, Davies VJ, Hogan VE, et al. OPA1 deficiency associated with increased autophagy in retinal ganglion cells in a murine model of dominant optic atrophy. *Invest Ophthalmol Vis Sci* 2009;50:2567-2571

Whitehead DL, Davies AD, Playfer JR, Turnbull CJ. Circadian rest-activity rhythm is altered in Parkinson's disease patients with hallucinations. *Mov Disord* 2008; 23:1137-1145.

Whitworth AJ, Pallanck LJ. The PINK1/Parkin pathway: a mitochondrial quality control system? *J Bioenerg Biomembr* 2009;41:499-503.

Willis GL. Parkinson's disease as a neuroendocrine disorder of circadian function: dopamine-melatonin imbalance and the visual system in the genesis and progression of the degenerative process. *Rev Neurosci* 2008a; 19:245-316.

Willis GL, Kelly AM, Kennedy GA. Compromised circadian function in Parkinson's disease: enucleation augments disease severity in the unilateral model. *Behav Brain Res* 2008b; 193:37-47.

Willis GL. Intraocular microinjections repair experimental Parkinson's disease. *Brain Res* 2008c; 1217:119-131.

Wisor JP, Edgar DM, Yesavage J, Ryan HS, McCormick CM, Lapustea N, et al. Sleep and circadian abnormalities in a transgenic mouse model of Alzheimer's disease: a role for cholinergic transmission. *Neuroscience* 2005; 131:375-385.

Witting W, Kwa IH, Eikelenboom P, Mirmiran M, Swaab DF. Alterations in the circadian rest-activity rhythm in aging and Alzheimer's disease. *Biol Psychiatry* 1990; 27:563-572.

Wostyn P, Audenaert K, De Deyn PP. Alzheimer's disease and glaucoma: is there a causal relationship? *Br J Ophthalmol* 2009; 93:1557-1559.

Wostyn P, Audenaert K, De Deyn PP. Alzheimer's disease: cerebral glaucoma? *Med Hypotheses* 2010; 74:973-977.

Wu YH, Swaab DF. The human pineal gland and melatonin in aging and Alzheimer's disease. *J Pineal Res* 2005;38:145-52.

Wu YH, Swaab DF. Disturbance and strategies for reactivation of the circadian rhythm system in aging and Alzheimer's disease. *Sleep Medicine* 2007a; 8: 623–636.

Wu YH, Zhou JN, Van Heerikhuize J, Jockers R, Swaab DF. Decreased MT1 melatonin receptor expression in the suprachiasmatic nucleus in aging and Alzheimer's disease. *Neurobiol Aging* 2007b; 28:1239-1247.

Wulff K, Porcheret K, Cussans E, Foster RG. Sleep and circadian rhythm disturbances: multiple genes and multiple phenotypes. *Curr Opin Genet Dev* 2009;19:237-46.

Yavas GF, Yilmaz O, Küsbeci T, Oztürk F. The effect of levodopa and dopamine agonists on optic nerve head in Parkinson disease. *Eur J Ophthalmol* 2007; 17:812-816.

Yesavage JA, Friedman L, Kraemer H, Tinklenberg JR, Salehi A, Noda A, et al. Sleep/wake disruption in Alzheimer's disease: APOE status and longitudinal course. *J Geriatr Psychiatry Neurol* 2004; 17:20-24.

Zaidi FH, Hull JT, Peirson SN, Wulff K, Aeschbach D, Gooley JJ, et al. Short-wavelength light sensitivity of circadian, pupillary, and visual awareness in humans lacking an outer retina. *Curr Biol* 2007; 17:2122-212.

Zavada A, Gordijn MC, Beersma DG, Daan S, Roenneberg T. Comparison of the Munich Chronotype Questionnaire with the Horne-Ostberg's Morningness-Eveningness Score. *Chronobiol Int* 2005;22:267-78.

Zhang X, Jones D, Gonzalez-Lima F. Neurodegeneration produced by rotenone in the mouse retina: A potential model to investigate environmental pesticide contributions to neurodegenerative diseases. *Journal of Toxicology and Environmental Health Part A* 2006; 69: 1681–1697.

Zhang DQ, Wong KY, Sollars PJ, Berson DM, Pickard GE, McMahon DG. Intraretinal signaling by ganglion cell photoreceptors to dopaminergic amacrine neurons. *Proc Natl Acad Sci U S A* 2008; 105:14181-14186.

Zhou JN, Liu RY, Kamphorst W, Hofman MA, Swaab DF. Early neuropathological Alzheimer's changes in aged individuals are accompanied by decreased cerebrospinal fluid melatonin levels. *J Pineal Res* 2003; 35:125-130.

Zhou QP, Jung L, Richards KC. The Management of Sleep and Circadian Disturbance in Patients with Dementia. *Curr Neurol Neurosci Rep* 2012 Feb 8. [Epub ahead of print]

Zucco G, Zeni MT, Perrone A, Piccolo I. Olfactory sensitivity in early-stage Parkinson patients affected by more marked unilateral disorder. *Percept Mot Skills* 2001;92:894-8.

Acknowledgments:

IRCCS Istituto delle Scienze Neurologiche, Dipartimento di Scienze Neurologiche, Università di Bologna, Bologna
Valerio Carelli
Roberto Gallassi
Luisa Sambati
Roberto Poda
Federico Oppi
Michelangelo Stanzani Maserati
Federica Provini
Fabio Pizza
Giuseppe Plazzi
Leonardo Caporali
Maria Stefania Bianconi
Paolo Martinelli
Giovanni Rizzo
Cesa Scaglione
Sabina Capellari
Sara Bonazza
Rocco Liguori
Maria Pia Giannoccaro
Pietro Cortelli
Giovanna Calandra-Buonaura
Piero Parchi

Studio Oculistico d'Azeglio, Bologna
Piero Barboni
Michele Carbonelli
Giacomo Savini

Dipartimento di Psicologia, Università di Bologna, Bologna
Vincenzo Natale

Dipartimento di Neuroscienze, Università di Parma
Pietro Avanzini
Gaetano Cantalupo

Department of Ophthalmology, Keck School of Medicine, University of Southern California, and Doheny Eye Institute, Los Angeles (CA), USA
Alfredo A Sadun
Fred N Ross-Cisneros
Kevin Tozer
Billy Pan

Department of Clinical Biochemistry, Bispebjerg Hospital and Rigshopitalet, University of Copenhagen, Denmark
Jens Hannibal
Birgitte Georg

……Un ringraziamento particolare a tutte le persone che mi sono state vicino sempre e nonostante tutto in questi anni, ai miei genitori che mi hanno sempre sostenuta ed incoraggiata, a Gaetano…….

ANNEX 1

doi:10.1093/brain/awq155 Brain 2010: 133; 2426–2438 | 2426

BRAIN
A JOURNAL OF NEUROLOGY

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

Chiara La Morgia,[1,*] Fred N. Ross-Cisneros,[2,*] Alfredo A. Sadun,[2] Jens Hannibal,[3] Alessandra Munarini,[4] Vilma Mantovani,[4] Piero Barboni,[1] Gaetano Cantalupo,[5] Kevin R. Tozer,[2] Elisa Sancisi,[1] Solange R. Salomao,[6] Milton N. Moraes,[6] Milton N. Moraes-Filho,[6] Steffen Heegaard,[7] Dan Milea,[8,9] Poul Kjer,[7] Pasquale Montagna[1] and Valerio Carelli[1,2]

1 Department of Neurological Sciences, University of Bologna, Bologna 40127, Italy
2 Department of Ophthalmology, Doheny Eye Institute, Keck School of Medicine, University of Southern California, Los Angeles 90033, CA, USA
3 Department of Clinical Biochemistry, Bispebjerg Hospital and Rigshopitalet, University of Copenhagen, Copenhagen 2400, Denmark
4 Biomedical Centre Applied Research CRBA, S.Orsola-Malpighi University Hospital, Bologna 40138, Italy
5 Department of Neuroscience, University of Parma, Parma 43125, Italy
6 Department of Ophthalmology, Federal University of São Paulo, UNIFESP, São Paulo 04023-900, Brazil
7 Department of Neuroscience and Pharmacology, Eye Pathology Section, University of Copenhagen, Copenhagen 2200, Denmark
8 Copenhagen University Hospital, Glostrup, Copenhagen 2600, Denmark
9 Department of Ophthalmology, Angers University Hospital, Angers 49033, France

*These authors contributed equally to this work.

Correspondence to: Valerio Carelli,
Department of Neurological Sciences,
University of Bologna,
Via Ugo Foscolo 7,
40123 Bologna, Italy
E-mail: valerio.carelli@unibo.it

Correspondence may also be addressed to: Alfredo A. Sadun,
E-mail: asadun@usc.edu

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

Received March 17, 2010. Revised April 15, 2010. Accepted May 12, 2010

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; Hatonen *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 monoaminoxidase 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®, 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 collotes 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. 5J68, 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 RGCs 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 hemiretinas (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 neuropathies 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

ID	Sex	Age	Mutation	Onset	Visual acuity	
					Oculus destrum	Oculus sinistrum
L1	M	32	11778/ND4 mtDNA (homoplasmic)	26	0.01	0.02
L2	M	31	11778/ND4 mtDNA (homoplasmic)	15	0.01	0.0125
L3	F	52	3460/ND1 mtDNA (homoplasmic)	12	0.05	0.02
L4	M	28	14484/ND6 mtDNA (heteroplasmic)	17	0.32	0.16
L5	M	26	3460/ND1 mtDNA (homoplasmic)	17	0.04	0.02
D1	F	22	Mut OPA1 (frame-shift/stop-codon) Exon 27; 2708-2711del (TTAG) (heterozygous)	6	0.16	0.16
D2	M	41	Mut OPA1 (splicing error) Exon 9; 984 + 3A>T (heterozygous)	1	0.05	0.05
D3	F	41	Mut OPA1 (missense) Exon 27; 2729T>A (Val910Asp) (heterozygous)	1	0.2	0.2
D4	M	45	Mut OPA1 (frame-shift/stop-codon) Exon 27; 2708-2711del (TTAG) (heterozygous)	<10	0.32	0.4

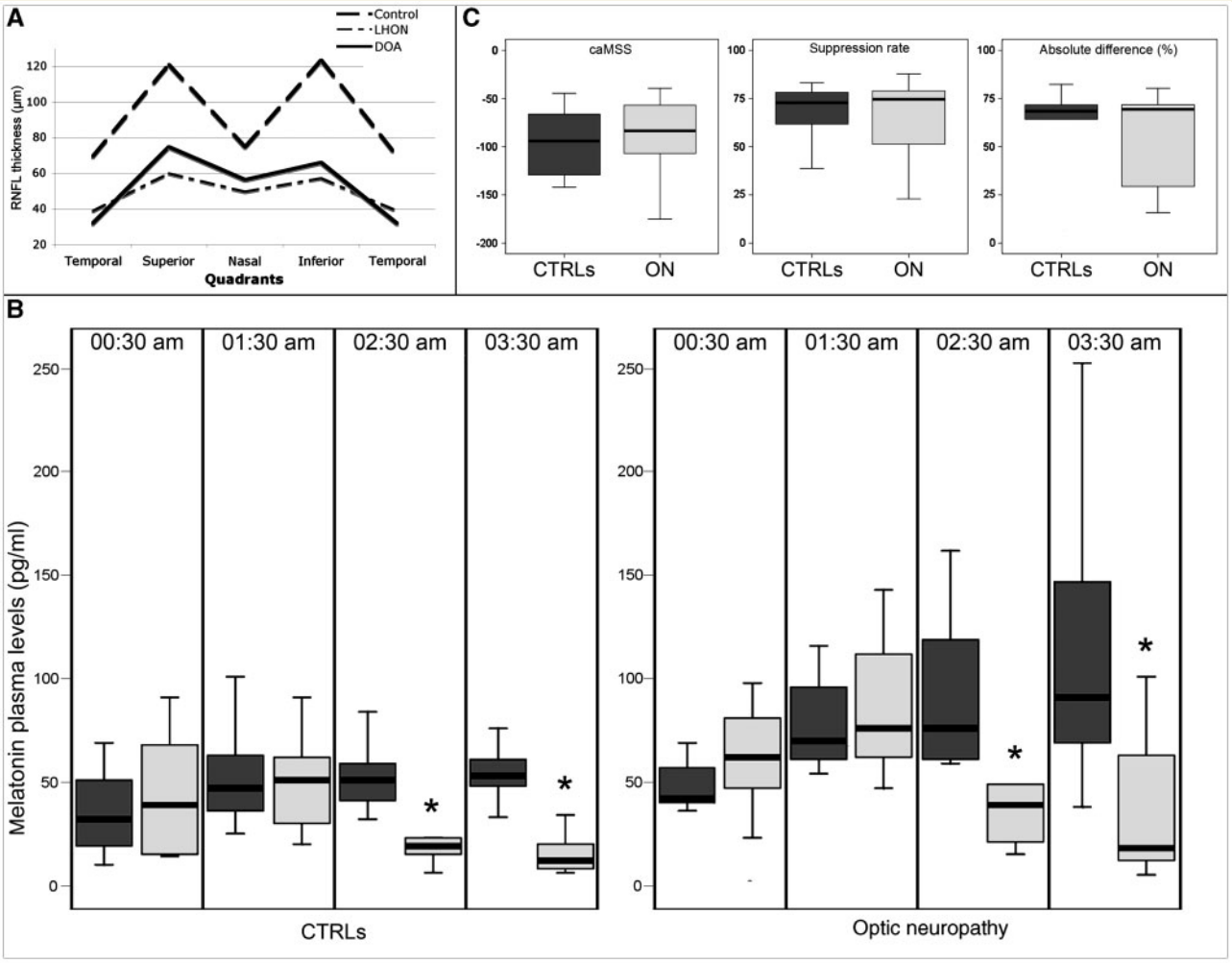


Figure 1 (**A**) Retinal nerve fibre layer thickness evaluated by optical coherence tomography in optic neuropathy and control subjects. Average retinal nerve fibre layer (RNFL) thicknesses of temporal, superior, nasal and inferior quadrants are shown for control, LHON and DOA subjects. Retinal nerve fibre layer thickness was significantly lower in optic neuropathy subjects compared with controls in each quadrant. There were no statistical differences between LHON and DOA subjects except for superior retinal nerve fibre layer thickness, which was significantly lower in LHON subjects ($P = 0.037$). The average retinal nerve fibre layer thickness was 97.3 μm ± 8.4 in controls, 52 μm ± 9 in LHON and 59.3 μm ± 5.8 in DOA subjects. (**B**) Melatonin suppression test results in control and optic neuropathy subjects. Box plots of melatonin plasma levels at the four sampling times (00:30, 01:30, 02:30, 03:30 am) are shown for the baseline (dark grey) and light suppression (light grey) nights in control (CTRLs; left panel) and optic neuropathy (right panel) subjects. In the control group, a significant difference in melatonin plasma levels between the baseline and the test night was evident for the third ($P = 0.000008$) and fourth samples ($P = 0.00015$); this difference was also evident in optic neuropathy subjects for the third ($P = 0.0018$) and fourth ($P = 0.0046$) samples. Asterisks indicate these significant differences. (**C**) Melatonin suppression scores for controls and optic neuropathy subjects. Box plots of control-adjusted melatonin suppression score (caMSS), suppression rate and absolute difference (%) scores are shown for control (dark grey) and optic neuropathy (ON; light grey) subjects in the left, middle and right panels, respectively. No statistical differences were evident between groups, demonstrating similar magnitude of light-induced melatonin suppression.

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 1 200 000 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 mRGCs are homogeneous in distribution over the 1040 mm^2 established total retinal surface (Curcio and Allen, 1990). Thus, quantitative evaluation by manual counting of mRGCs resulted in a mean density of ~18 cells/mm^2 for Control 1 and 13 cells/mm^2 for Control 2, whereas the mean density was 8 cells/mm^2 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 (Baver *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 sectorial 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 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

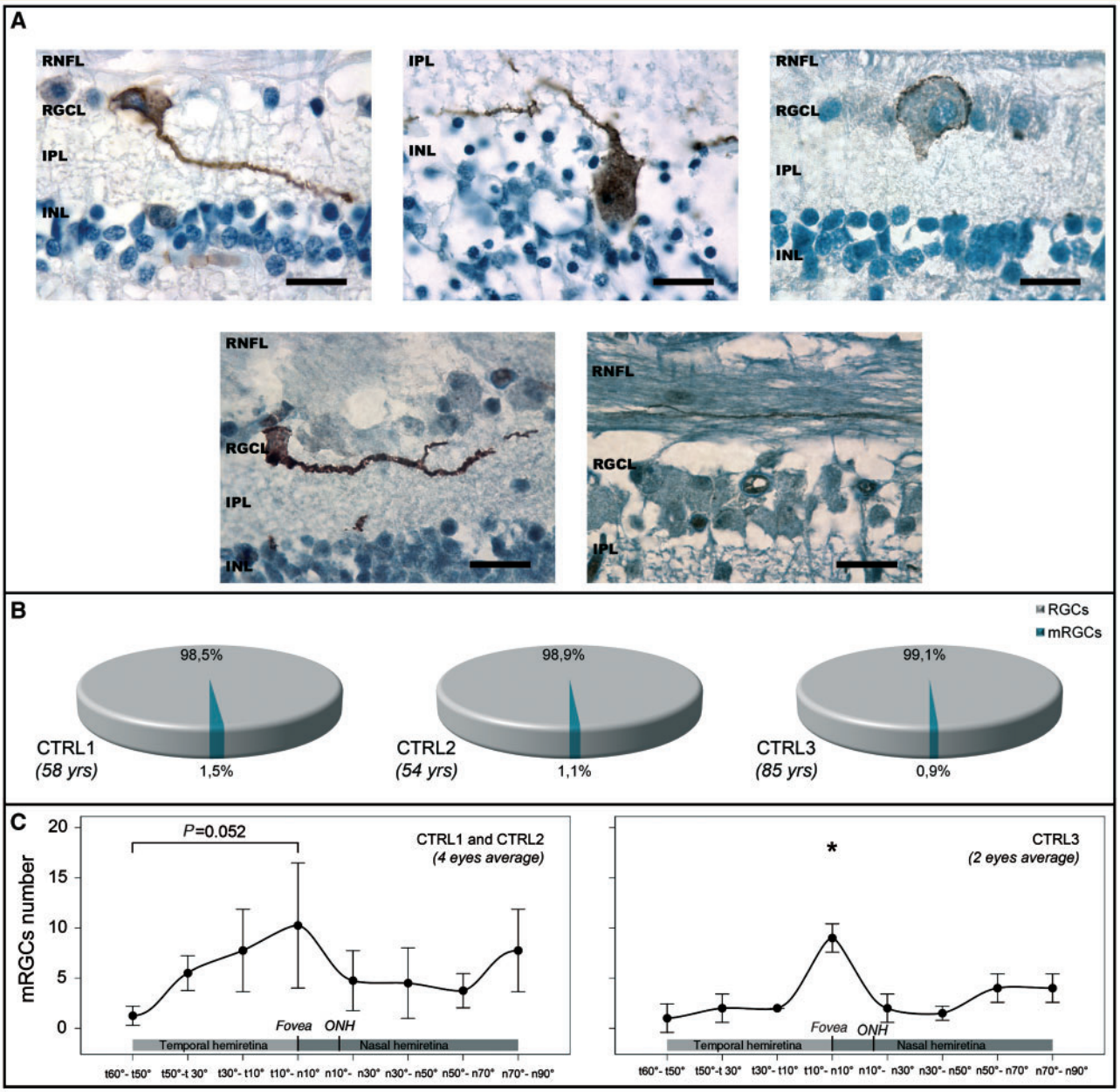


Figure 2 (**A**) Melanopsin retinal ganglion cells in control retinas. Upper line: (left) one example of a brown-stained mRGC located in the RGC layer is shown including the large cell body with the nucleus and a dendrite running at the border of the inner nuclear layer; (middle) a brown-stained mRGC 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 ($t10°–n10°$) 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 ($t10°–n10°$) is also greater and significantly different ($P = 0.05$) with respect to all sectors except for $n50°–n70°$ and $n70°–n90°$ ($t10°–n10°$ versus: $t60°–t50°$ $P = 0.01$; $t50°–t30°$ $P = 0.024$; $t30°–t10°$ $P = 0.024$; $n10°–n30°$ $P = 0.024$; $n30°–n50°$ $P = 0.015$). RNFL = retinal nerve fibre layer; RGCL = retinal ganglion cell layer; IPL = inner plexiform layer; INL = inner nuclear layer.

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

ID	Sex	Age	Mutation	Onset	Visual acuity		Pupillary light reflex		Visual fields		Fundus		Scanning laser polarimetry
					OD	OS	OD	OS	OD	OS	OD	OS	
Mild LHON	M	59	11778/ND4 mtDNA (homoplasmic)	51	<0.01 (light perception)	<0.01 (light perception)	Normal	Normal	Central scotoma	Central scotoma	Temporal atrophy	Temporal atrophy (more evident than OD)	Moderate nerve fibre loss
Severe LHON	M	52	11778/ND4 mtDNA (homoplasmic)	27	0.01 (hand motion)	0.01 (hand motion)	Sluggish	Sluggish	Large central scotoma	Large central scotoma	Complete optic atrophy	Complete optic atrophy	Severe nerve fibre loss
DOA	M	84	Mut. OPA1 Exon 28; 2826delT (p.V942fsX967)	Childhood	0.1	Counting fingers	Normal	Normal	Central scotoma	Central scotoma	Complete optic atrophy	Complete optic atrophy	n.a.

n.a. = not available; OD = oculus destrum; OS = oculus sinistrum.

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 ~300 000 axons for the mild LHON (74% loss as compared with 1 200 000 axons in normal eyes) and ~22 500 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 $t60°–t50°$, $t50°–t30°$ and $n50°–n70°$ ($P = 0.002$, 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.

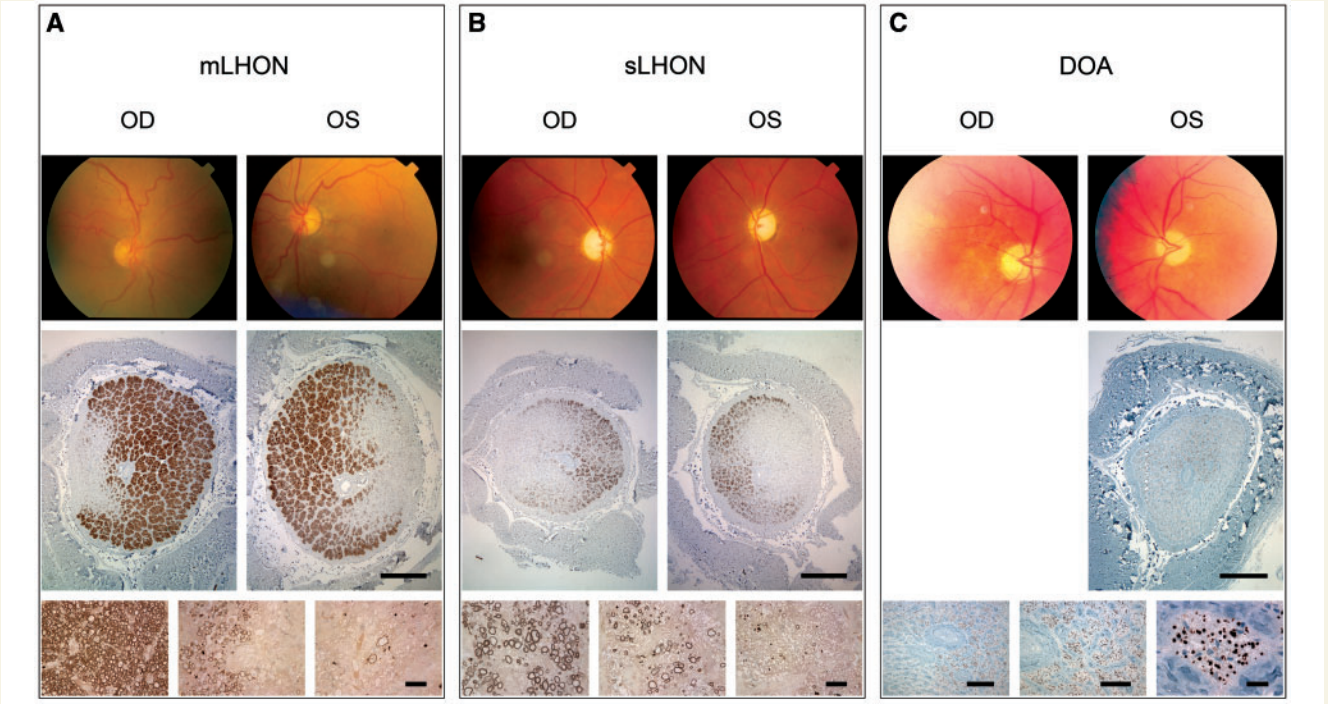


Figure 3 (**A**) Ocular findings in the mild LHON case (mLHON). Upper line, on the left the oculus destrum (OD) fundus with moderate temporal pallor of the optic disc is shown; on the right the oculus sinistrum (OS) fundus is characterized by a more severe sectorial temporal pallor of the optic disc. Middle line, on the left oculus destrum optic nerve cross-section shows severe depletion of axonal bundles involving the temporal fibres; on the right oculus sinistrum optic nerve cross-section reveals more extended depletion of axonal bundles involving the temporal fibres (scale bar represents 1 mm). Lower line, on the left, high-magnification images of nasal axonal bundles demonstrate high, virtually normal axonal density, whereas in the middle the transition zone is characterized by intermediate axonal density, and on the right, the temporal sector shows low axonal density (scale bar represents 20 μm). Axons are identified as myelin profiles stained by paraphenylenediamine on semithin sections cut from plastic-embedded tissue (see 'Materials and methods' section). (**B**) Ocular findings in the severe optic atrophy case (sLHON). Upper line, funduscopic images show complete optic atrophy on both oculus destrum (left) and oculus sinistrum (right). Middle line, oculus destrum (left) and oculus sinistrum (right) optic nerve cross-sections reveal diffuse and severe depletion of axonal bundles involving the entire optic nerve with some preservation of the nasal bundles (scale bar represents 1 mm). Lower line, high-magnification images of nasal axonal bundles demonstrate depleted axonal density (left), whereas the transition zone reveals low axonal density (middle) and the temporal sector shows very low axonal density (scale bar represents 20 μm). Axons are identified as myelin profiles stained by paraphenylenediamine on semithin sections cut from plastic-embedded tissue (see 'Materials and methods' section). (**C**) Ocular findings in the DOA case. Upper line, funduscopic images show complete optic atrophy on both oculus destrum (left) and oculus sinistrum (right). Middle line, OS optic nerve cross-section shows diffuse and very severe depletion of axonal bundles involving the entire optic nerve (scale bar represents 1 mm). Lower line, images at increasing magnification show the axonal depletion of spared bundles in the central area of the optic nerve cross-section (scale bars represent, respectively, 100, 50 and 20 μm). Axons are identified as axoplasm immunostained for neurofilaments on sections cut from paraffin tissue blocks (see 'Materials and methods' section).

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

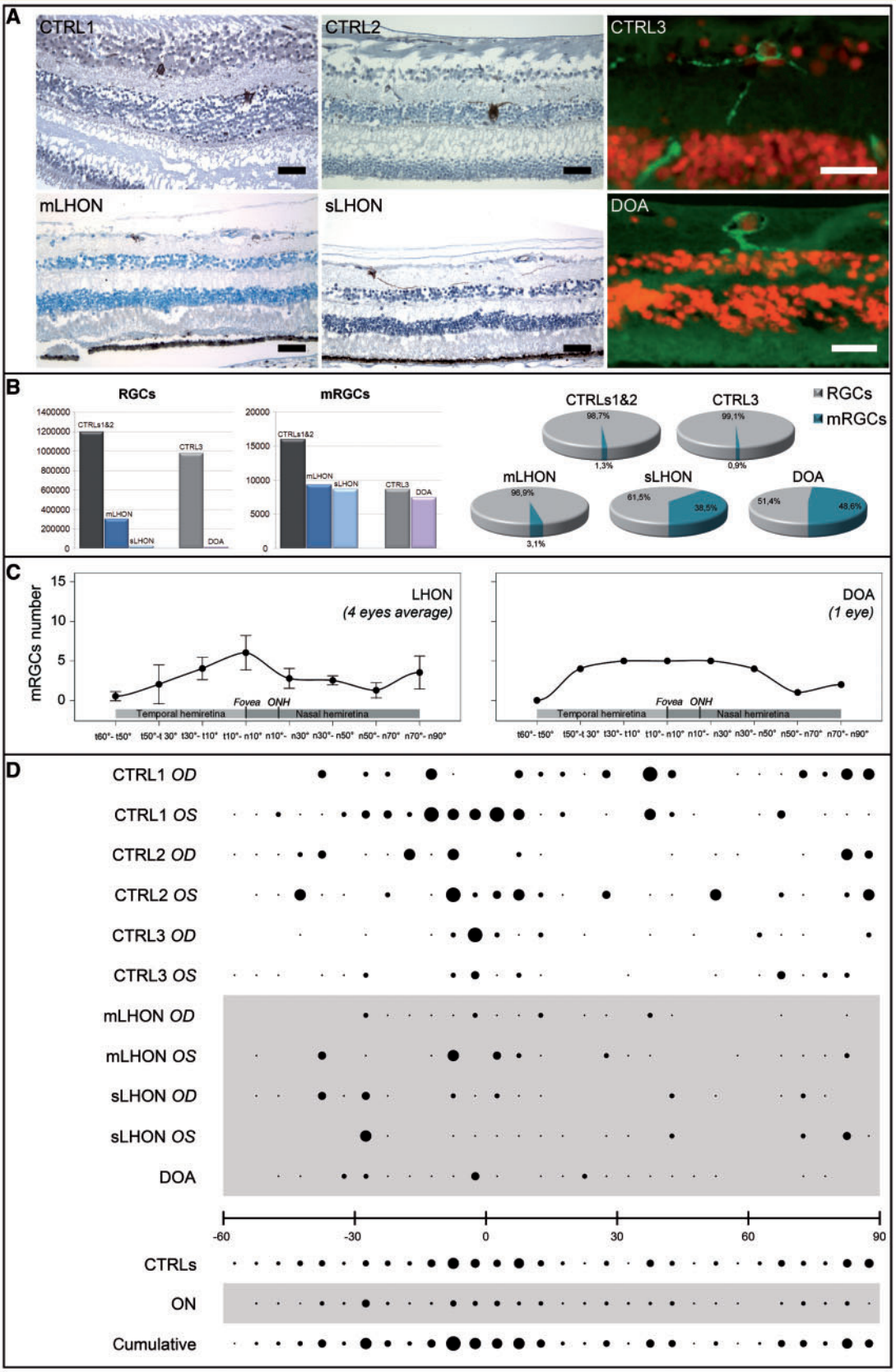


Figure 4 (**A**) Melanopsin cells in control and optic neuropathy retinas. In the upper line, examples of brown-stained mRGCs located both in the RGC layer and in the inner nuclear layer for all three controls are provided (scale bar represents 60 μm). The Control 3 sections were stained by immunofluorescence 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

Continued

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 photoentrainment 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 hemiretinas, centred on the fovea. Mean number of mRGCs in parafoveal sector ($t10°–n10°$) is greater in the two LHON subjects, being significantly different compared with the sectors $t60°–t50°$, $t50°–t30°$ and $n50°–n70°$ ($P = 0.002$, 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 transection 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 unmyelinated 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.

Acknowledgements

The authors would like to express their gratitude to Dr Divya Aggarwal, Craig Robison, Ernesto Barron and Alice Leith for their technical assistance, the SOA-BR family who kindly gave us permission to examine the ocular tissues from their relatives, and the Lions Eye Bank of Oregon, USA for supplying the control ocular tissues.

Funding

This project was supported, in part, by a Telethon-Italy grant # GGP06233 (V.C.), the International Foundation for Optic Nerve Diseases (F.N.R.-C., A.A.S., V.C. and S.R.S.), Research to Prevent Blindness (F.N.R.-C., A.A.S. and K.R.T.), Struggling Within Leber's Foundation (F.N.R.-C., A.A.S. and K.R.T.), National Institutes of Health grant EY03040 (F.N.R.-C., A.A.S. and K.R.T.) and the Ermian Foundation (F.N.R.-C., A.A.S. and K.R.T.).

Supplementary material

Supplementary material is available at *Brain* online.

References

Baver SB, Pickard GE, Sollars PJ, Pickard GE. Two types of melanopsin retinal ganglion cell differentially innervate the hypothalamic suprachiasmatic nucleus and the olivary pretectal nucleus. Eur J Neurosci 2008; 27: 1763–70.

Berson DM, Dunn FA, Takao M. Phototransduction by retinal ganglion cells that set the circadian clock. Science 2002; 295: 1070–3.

Bose S, Dhillon N, Ross-Cisneros FN, Carelli V. Relative post-mortem sparing of afferent pupil fibers in a patient with 3460 Leber's hereditary optic neuropathy. Graefes Arch Clin Exp Ophthalmol 2005; 243: 1175–9.

Brainard GC, Hanifin JP, Greeson JM, Byrne B, Glickman G, Gerner E, et al. Action spectrum for melatonin regulation in humans: evidence for a novel circadian photoreceptor. J Neurosci 2001; 21: 6405–12.

Bremner FD, Shallo-Hoffmann J, Riordan-Eva P, Smith SE. Comparing pupil function with visual function in patients with Leber's hereditary optic neuropathy. Invest Ophthalmol Vis Sci 1999; 40: 2528–34.

Bremner FD, Tomlin EA, Shallo-Hoffmann J, Votruba M, Smith SE. The pupil in dominant optic atrophy. Invest Ophthalmol Vis Sci 2001; 42: 675–8.

Carelli V, Achilli A, Valentino ML, Rengo C, Semino O, Pala M, et al. Haplogroup effects and recombination of mitochondrial DNA: novel clues from the analysis of Leber hereditary optic neuropathy pedigrees. Am J Hum Genet 2006; 78: 564–74.

Carelli V, Ross-Cisneros FN, Sadun AA. Mitochondrial dysfunction as a cause of optic neuropathies. Prog Retin Eye Res 2004; 23: 53–89.

Chambille I, Serviere J. Neurotoxic effects of neonatal injections of monosodium L-glutamate (L-MSG) on the retinal ganglion cell layer of the golden hamster: anatomical and functional consequences on the circadian system. J Comp Neurol 1993; 338: 67–82.

Curcio CA, Allen KA. Topography of ganglion cells in human retina. J Comp Neurol 1990; 300: 5–25.

Czeisler CA, Shanahan TL, Klerman EB, Martens H, Brotman DJ, Emens JS, et al. Suppression of melatonin secretion in some blind patients by exposure to bright light. N Engl J Med 1995; 332: 6–11.

Dacey DM, Liao HW, Peterson BB, Robinson FR, Smith VC, Pokorny J, et al. Melanopsin-expressing ganglion cells in primate retina signal colour and irradiance and project to the LGN. Nature 2005; 433: 749–54.

Davies VJ, Hollins AJ, Piechota MJ, Yip W, Davies JR, White KE, et al. Opa1 deficiency in a mouse model of autosomal dominant optic atrophy impairs mitochondrial morphology, optic nerve structure and visual function. Hum Mol Genet 2007; 16: 1307–18.

Foster RG, Provencio I, Hudson D, Fiske S, De GW, Menaker M. Circadian photoreception in the retinally degenerate mouse (rd/rd). J Comp Physiol A 1991; 169: 39–50.

Freedman MS, Lucas RJ, Soni B, von Schantz M, Muñoz M, David-Gray Z, et al. Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors. Science 1999; 284: 502–4.

Fu Y, Liao HW, Do MT, Yau KW. Non-image-forming ocular photoreception in vertebrates. Curr Opin Neurobiol 2005; 15: 415–22.

Goz D, Studholme K, Lappi DA, Rollag MD, Provencio I, Morin LP. Targeted destruction of photosensitive retinal ganglion cells with a saporin conjugate alters the effects of light on mouse circadian rhythms. PLoS One 2008; 3: e3153.

Hannibal J, Fahrenkrug J. Neuronal input pathways to the brain's biological clock and their functional significance. Adv Anat Embryol Cell Biol 2006; 182: 1–71.

Hannibal J, Hindersson P, Knudsen SM, Georg B, Fahrenkrug J. The photopigment melanopsin is exclusively present in pituitary adenylate cyclase-activating polypeptide-containing retinal ganglion cells of the retinohypothalamic tract. J Neurosci 2002; 22: RC191.

Hannibal J, Hindersson P, Ostergaard J, Georg B, Heegaard S, Larsen PJ, et al. Melanopsin is expressed in PACAP-containing retinal ganglion cells of the human retinohypothalamic tract. Invest Ophthalmol Vis Sci 2004; 45: 4202–9.

Hannibal J, Vrang N, Card JP, Fahrenkrug J. Light-dependent induction of cFos during subjective day and night in PACAP-containing ganglion cells of the retinohypothalamic tract. J Biol Rhythms 2001; 16: 457–470.

Hatonen T, Laakso ML, Heiskala H, ila-Johansson A, Sainio K, Santavuori P. Bright light suppresses melatonin in blind patients with neuronal ceroid-lipofuscinoses. Neurology 1998; 50: 1445–50.

Hattar S, Kumar M, Park A, Tong P, Tung J, Yau KW, et al. Central projections of melanopsin-expressing retinal ganglion cells in the mouse. J Comp Neurol 2006; 497: 326–49.

Hattar S, Liao HW, Takao M, Berson DM, Yau KW. Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science 2002; 295: 1065–70.

Hebert M, Martin SK, Lee C, Eastman CI. The effects of prior light history on the suppression of melatonin by light in humans. J Pineal Res 2002; 33: 198–203.

Johnson BM, Miao M, Sadun AA. Age-related decline of human optic nerve axon populations. Age 1987; 10: 5–9.

Jusuf PR, Lee SC, Hannibal J, Grunert U. Characterization and synaptic connectivity of melanopsin-containing ganglion cells in the primate retina. Eur J Neurosci 2007; 26: 2906–21.

Kawasaki A, Herbst K, Sander B, Milea D. Selective wavelength pupillometry in Leber's hereditary optic neuropathy. Clin Exp Ophthalmol 2010; 38: 322–4.

Kjer P. Infantile optic atrophy with dominant mode of inheritance: a clinical and genetic study of 19 Danish families. Acta Ophthalmol Suppl 1959; 164: 1–147.

Kjer P, Jensen OA, Klinken L. Histopathology of eye, optic nerve and brain in a case of dominant optic atrophy. Acta Ophthalmol 1983; 61: 300–12.

Klein DC, Weller JL. Rapid light-induced decrease in pineal serotonin N-acetyltransferase activity. Science 1972; 177: 532–3.

Li RS, Chen BY, Tay DK, Chan HH, Pu ML, So KF. Melanopsin-expressing retinal ganglion cells are more injury-resistant in a chronic ocular hypertension model. Invest Ophthalmol Vis Sci 2006; 47: 2951–8.

Lubkin V, Beizai P, Sadun AA. The eye as metronome of the body. Surv Ophthalmol 2002; 47: 17–26.

Mure LS, Rieux C, Hattar S, Cooper HM. Melanopsin-dependent nonvisual responses: evidence for photopigment bistability in vivo. J Biol Rhythms 2007; 22: 411–24.

Osborne NN, Li GY, Ji D, Mortiboys HJ, Jackson S. Light affects mitochondria to cause apoptosis to cultured cells: possible relevance to ganglion cell death in certain optic neuropathies. J Neurochem 2008; 105: 2013–28.

Peirson S, Foster RG. Melanopsin: another way of signaling light. Neuron 2006; 49: 331–9.

Perez-Rico C, de la Villa P, Blanco R, Germain F, Paz-Moreno J, rribas-Gomez I. Alterations in nocturnal melatonin secretion in patients with optic neuropathies. Arch Soc Esp Oftalmol 2009; 84: 251–7.

Robinson GA, Madison RD. Axotomized mouse retinal ganglion cells containing melanopsin show enhanced survival, but not enhanced axon regrowth into a peripheral nerve graft. Vision Res 2004; 44: 2667–74.

Sadun AA, Schaechter JD, Smith LE. A retinohypothalamic pathway in man: light mediation of circadian rhythms. Brain Res 1984; 302: 371–7.

Sadun AA, Carelli V, Salomao SR, Berezovsky A, Quiros PA, Sadun F, et al. Extensive investigation of a large Brazilian pedigree of 11778/haplogroup J Leber hereditary optic neuropathy. Am J Ophthalmol 2003; 136: 231–8.

Sadun AA, Win PH, Ross-Cisneros FN, Walker SO, Carelli V. Leber's hereditary optic neuropathy differentially affects smaller axons in the optic nerve. Trans Am Ophthalmol Soc 2000; 98: 223–32.

Semo M, Lupi D, Peirson SN, Butler JN, Foster RG. Light-induced c-fos in melanopsin retinal ganglion cells of young and aged rodless/coneless (rd/rd cl) mice. Eur J Neurosci 2003; 18: 3007–17.

Semo M, Munoz LM, Foster RG, Jeffery G. Melanopsin (Opn4) positive cells in the cat retina are randomly distributed across the ganglion cell layer. Vis Neurosci 2005; 22: 111–6.

Tenhula WN, Xu SZ, Madigan MC, Heller K, Freeman WR, Sadun AA. Morphometric comparisons of optic nerve axon loss in acquired immunodeficiency syndrome. Am J Ophthalmol 1992; 113: 14–20.

Thiselton DL, Alexander C, Taanman JW, Brooks S, Rosenberg T, Eiberg H, et al. A comprehensive survey of mutations in the OPA1 gene in patients with autosomal dominant optic atrophy. Invest Ophthalmol Vis Sci 2002; 43: 1715–24.

Viney TJ, Balint K, Hillier D, Brooks S, Rosenberg T, Eiberg H, et al. Local retinal circuits of melanopsin-containing ganglion cells identified by transsynaptic viral tracing. Curr Biol 2007; 17: 981–8.

von Bussmann KA, Garey LJ, Jen LS. Injury-resistant retinal ganglion cells that are rich in cytochrome oxidase. Neuroreport 1993; 4: 247–50.

Vugler AA, Redgrave P, Semo M, Lawrence J, Greenwood J, Coffey PJ. Dopamine neurones form a discrete plexus with melanopsin cells in normal and degenerating retina. Exp Neurol 2007; 205: 26–35.

Wakakura M, Yokoe J. Evidence for preserved direct pupillary light response in Leber's hereditary optic neuropathy. Br J Ophthalmol 1995; 79: 442–6.

Wee R, Van Gelder RN. Sleep disturbances in young subjects with visual dysfunction. Ophthalmology 2004; 111: 297–302.

ANNEX 2

Vision Research 51 (2011) 296–302

Contents lists available at ScienceDirect

Vision Research

journal homepage: www.elsevier.com/locate/visres

Review

Melanopsin-expressing retinal ganglion cells: implications for human diseases

Chiara La Morgia [a,*], Fred N. Ross-Cisneros [b], Jens Hannibal [c], Pasquale Montagna [a], Alfredo A. Sadun [b], Valerio Carelli [a,b]

[a] Department of Neurological Sciences, University of Bologna, Bologna, Italy
[b] Doheny Eye Institute, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA
[c] Department of Clinical Biochemistry, Bispebjerg Hospital, University of Copenhagen, Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 15 June 2010
Received in revised form 27 July 2010
Available online 4 August 2010

Keywords:
Melanopsin
Hereditary optic neuropathies
Circadian
Aging
Neurodegeneration
Mitochondria

ABSTRACT

In the last decade, there was the seminal discovery of melanopsin-expressing retinal ganglion cells (mRGCs) as a new class of photoreceptors that subserve the photoentrainment of circadian rhythms and other non-image forming functions of the eye. Since then, there has been a growing research interest on these cells, mainly focused on animal models. Only recently, a few studies have started to address the relevance of the mRGC system in humans and related diseases.

We recently discovered that mRGCs resist neurodegeneration in two inherited mitochondrial disorders that cause blindness, i.e. Leber hereditary optic neuropathy and dominant optic atrophy. The mechanism leading to mRGCs sparing in these blinding disorders, characterized by extensive and selective loss of RGCs, is currently unknown and under investigation. Other studies reported on mRGCs in glaucoma, on genetic variation of the melanopsin gene (*OPN4*) in seasonal affective disorder and on the role of mRGCs in migraineous photophobia. Our own data and studies from others have shown a significant reduction of mRGCs with aging.

We anticipate that these studies will lead to many other investigations addressing the role of mRGCs and circadian photoreception in the pathogenesis of circadian and sleep abnormalities in neurodegenerative disorders.

1. Introduction

Many physiological functions in the human body are characterized by rhythmic changes within a 24 h period generated by the circadian pacemaker, the hypothalamic suprachiasmatic nucleus (SCN) (Reppert & Weaver, 2002). Studies on the mechanisms of photoentrainment, i.e. how light adjusts the phase of the circadian rhythms to the astronomical day length, led to the breakthrough discovery in 2002 of a third, non-rod non-cone photoreceptor in the mammalian retina, expressing a photopigment named melanopsin (Berson, Dunn, & Takao, 2002; Hannibal, Hindersson, Knudsen, Georg, & Fahrenkrug, 2002; Hattar, Liao, Takao, Berson, & Yau, 2002; Provencio, Rollag, & Castrucci, 2002). Melanopsin was identified in a subtype of retinal ganglion cells (RGCs), which constitute about 1% of total RGCs (Berson et al., 2002; Dacey et al., 2005; Hannibal, Hindersson, Nevo, & Fahrenkrug, 2002; Hannibal et al., 2004; Hattar et al., 2002; Provencio et al., 2000) (Fig. 1).

The melanopsin-expressing RGCs (mRGCs) constitute the monosynaptic neuronal pathway identified in mammals by Moore and Lenn (1972) and Hendrickson, Wagoner, and Cowan (1972) and later described in humans referred to as the retinohypothalamic tract (RHT) (Berson et al., 2002; Dacey et al., 2005; Hannibal et al., 2002; Hattar et al., 2002; Sadun, Schaechter, & Smith, 1984). It is now well established that mRGCs, through the RHT, mediate non-image forming functions of the eye, including the photoentrainment of circadian rhythms to the light–dark cycle (Guler et al., 2008; Hatori et al., 2008).

The mRGCs are characterized by unique properties being intrinsically photosensitive due to the expression of melanopsin, even in the absence of any other retinal input (Berson et al., 2002; Hattar et al., 2002; Lucas et al., 2003). This property allows these cells to absorb and process light whereas the other RGCs may suffer possible damage when exposed to direct light energy (Osborne, Li, Ji, Mortiboys, & Jackson, 2008; Tu et al., 2005). The mRGCs are maximally sensitive to short-wavelength blue light (between 459 and 483 nm). Melanopsin is a bistable photopigment, allowing mRGCs to regenerate their chromophore without involving other cells, similar to the photoreceptors of invertebrates (Fu et al., 2005; Mure, Rieux, Hattar, & Cooper, 2007). In fact, the phylogenetic origin of mRGCs is very ancient and relates to invertebrate rhabdomeric photoreception, which predates the development of the eye as the organ for vision (Peirson, Halford, & Foster, 2009). Thus, a long-lasting evolutionary pressure conserved this photoreceptive system, possibly selecting its intrinsic robustness. Moreover,

* Corresponding author. Address: Department of Neurological Sciences, University of Bologna, Via Ugo Foscolo 7, 40127 Bologna, Italy. Fax: +39 0512092751.
E-mail address: chiaralamorgia@gmail.com (C. La Morgia).

0042-6989/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.visres.2010.07.023

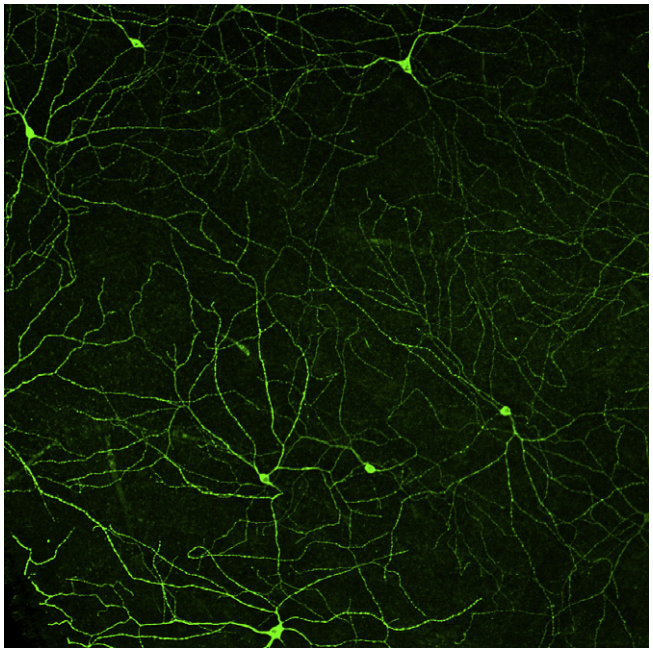


Fig. 1. Melanopsin expression in a subset of retinal ganglion cells in the human retina. A flat–mount preparation from a normal part of human retina removed due to cancer shows melanopsin immunoreactivity in the membrane of the soma and processes of approximately 1% of retinal ganglion cells forming a photosentitive network covering the entire retinal surface.

mRGCs provide the earliest light detection in mammals being functionally active at day 0 postnatal, highlighting the importance of their function in the developing retina (Hannibal & Fahrenkrug, 2004; Tarttelin et al., 2003). Finally, there is now growing evidence that mRGCs project to many areas of the brain serving many non-visual forming functions besides photoentrainment of circadian rhythms, such as regulation of the pupillary light reflex, melatonin secretion and its suppression by light, masking behavior, cognition, wakefulness and sleep (Berson, 2003; Dijk & Archer 2009; Lupi, Oster, Thompson, & Foster, 2008; Tsai et al., 2009). These many functions served by mRGCs are mirrored by their projections, which not only include the SCN, but also other hypothalamic nuclei, such as the ventro-lateral preoptic nucleus that contains sleep active neurons, and the subparaventricular zone involved in temperature and hormone regulation (Saper, Lu, Chou, & Gooley, 2005). The mRGCs also project to the olivary pretectal nuclei, constituting the afferent arm of the pupillary light response, the lateral habenula, a relay site between limbic and striatal areas, the amygdala involved in regulation of emotions and the intergeniculate leaflet of the thalamus playing a crucial role in mediating the non-photic regulation of circadian rhythms (Berson, 2003; Hannibal et al., 2004; Hattar et al., 2006; Vandewalle, Maquet, & Dijk, 2009). The widespread impact of light on the brain also includes the wavelength-dependent activation of alertness-related subcortical structures such as the brainstem, and in particular the locus coeruleus, as documented by functional-MRI studies (Vandewalle et al., 2007a; Vandewalle et al., 2007b). These cells also provide innervation to areas of the brain more typically characterized as crucial to vision, such as the lateral geniculate nucleus and the superior colliculus (Dacey et al., 2005; Hannibal & Fahrenkrug, 2006; Hattar et al., 2006).

Thus, the recent identification of mRGCs came as an important discovery that contributes greatly to our knowledge of retinal function, and it has fueled numerous studies aimed at delineating details of this cellular system with particular reference to chronobiology. The majority of studies have taken advantage of animal models by manipulating their genetic expression to define the physiology of mRGCs. Some of these studies took advantage of animal models recapitulating human diseases to infer how pathogenic mechanisms may involve mRGCs (Vugler, Semo, Joseph, & Jeffery, 2008). However, to date, only a few studies investigated the mRGCs-system directly in humans or in human pathology (Dacey et al., 2005; Hannibal et al., 2004; Vugler et al., 2007).

2. Retinal pathology and light-suppression of melatonin led to the discovery of mRGCs

The discovery of mRGCs came from the observation that mice with extensive degeneration of rods and cones, such as homozygous (*rd/rd*) mice lacking rods or mice lacking all functional rod and cone photoreceptors (*rd/rd cl*) were still capable of normal circadian photoentrainment (Foster et al., 1991; Freedman et al., 1999; Lucas, Freedman, Muñoz, Garcia-Fernández, & Foster, 1999). Moreover, a few studies of human blind subjects with mixed pathology of rods and cones or optic neuropathy documented the preservation of the light-induced melatonin suppression response indicating the existence of a different light perception system (Czeisler et al., 1995; Hätönen et al., 1998). An extreme example on the same theme is the completely blind mole rat *Spalax ehrenberghi*. This animal, despite the severe regression of the conventional visual pathways is still able to photoentrain its circadian rhythms. In fact, there are approximately 900 RGCs still existent in the mole rat despite its rudimentary subcutaneous eyes and most of these project to structures in the brain related to circadian photoreception (Cooper, Herbin, & Nevo, 1993). In this animal David-Gray and coauthors isolated a cone-like photopigment, purported to contribute to non-image forming functions (David-Gray, Janssen, DeGrip, Nevo, & Foster, 1998). Later melanopsin has been recognized as a major pigment in this species (Hannibal, Hindersson, Nevo, & Fahrenkrug, 2002).

Along the same lines, studies on the action spectrum of light-induced melatonin suppression response in normal human subjects led to the presumption that a short-wavelength pigment, clearly different from the photopigments of the classical scotopic and photopic visual systems, was crucial to mediate this response (Brainard et al., 2001; Thapan, Arendt, & Skene, 2001). All these observations were key in the discovery of mRGCs as a new class of photoreceptors mediating non-image forming functions of the eye (Berson et al., 2002; Hattar et al., 2002).

3. Mitochondrial optic neuropathies: the right model to study abnormal circadian photoreception?

Human blind subjects have been always considered a useful model for the study of circadian rhythm disorders (Sack & Lewy, 2001). However, in most studies, subjects enrolled were affected by heterogeneous retinal pathology and the occurrence of circadian rhythms misalignment was not considered with respect to the type of retinal disease. Based on the scattered data available, the occurrence of circadian photoentraiment disturbances seemed to be determined by the absence of light perception. In fact, the vast majority (77%) of blind subjects with light perception have normally entrained circadian rhythms, suggesting a preservation of the mRGCs–RHT–SCN circuit. In contradistinction, most of the subjects without light perception, especially those with ocular enucleation, have abnormal circadian photoentrainment (Skene & Arendt, 2007). Thus, the presence of circadian rhythm disturbances seemed to be related to the severity as well as the type of ocular disease (Wee & Van Gelder, 2004).

Diseases of the outer retina, selectively affecting rods and cones, and thus sparing the mRGCs, were particularly useful to identify the existence of mRGCs. Similarly, we thought that genetically

determined neurodegenerative optic neuropathies, selectively affecting RGCs, would be a good model to study the possible occurrence of abnormalities in circadian photoentrainment. Two such disorders, i.e. Leber hereditary optic neuropathy (LHON) and dominant optic atrophy (DOA) are in fact characterized by the selective loss of RGCs due to mitochondrial dysfunction (Carelli, Ross-Cisneros, & Sadun, 2004). Thus, we studied the mRGCs–RHT–SCN system in LHON and DOA using both a functional, i.e. melatonin suppression test by light, and a histological approach. We demonstrated that the mRGCs–RHT–SCN system is substantially unaffected in these visually impaired patients as they maintain the light-induced suppression of melatonin secretion. Furthermore, our histological investigation on post-mortem retinal and optic nerve specimens from LHON and DOA cases demonstrated a relative sparing of mRGCs (Fig. 2) leading to the unpredicted conclusion that these cells are resistant to neurodegeneration in mitochondrial optic neuropathies (La Morgia et al., 2010). These studies suggest the preservation of circadian photoentrainment in these patients.

Our findings are compatible with at least two observations. First, despite LHON and DOA patients are severely visually impaired, they usually maintain light perception vision (Carelli et al., 2004). Second, LHON and DOA patients have also preserved pupillary light response (Bremner, Tomlin, Shallo-Hoffmann, Votruba, & Smith, 2001; Wakakura & Yokoe, 1995), which can now be explained by our findings of mRGCs sparing. We had previously shown, in a post-mortem study of a single LHON case with extremely severe optic atrophy, spared axons leaving the optic chiasm and projecting to the pretectum (Bose, Dhillon, Ross-Cisneros, & Carelli, 2005; Sadun, Win, Ross-Cisneros, Walker, & Carelli, 2000). Also mice with rotenone-induced optic neuropathy, a model for LHON, preserve the pupillary light reflex, suggesting selective sparing of mRGCs (Zhang, Jones, & Gonzalez-Lima, 2006). Further indirect confirmation of our results is provided by a single case study of Kawasaki and coauthors who demonstrated, using selective wavelength pupillometry in a case of LHON, that the pupil response, driven by mRGCs, was spared (Kawasaki, Herbst, Sander, & Milea, 2010). Furthermore, Pérez-Rico and coauthors (2009) demonstrated, that 5 out of 10 blind subjects maintaining the light-induced melatonin suppression response included 2 DOA subjects.

Our study raises the question of how mRGCs are resistant to mitochondrial dysfunction. A review of the literature provides a body of supporting evidence for the resistance of these cells to different kind of injuries. Three studies on the dying-back model of RGCs after optic nerve transection are revealing. The first study showed that a small subset (about 1%) of surviving RGCs with large bodies intensively stained with cytochrome c oxidase (COX; mitochondrial respiratory complex IV) (von Bussmann, Garey, & Jen, 1993); their size and number would be most consistent with mRGCs. Preliminary data from our laboratory confirm that mRGCs are intensively stained with both mitochondrial and COX antibodies, suggesting an abundant mitochondrial population within these cells (Carla Giordano, unpublished results). A more recent study demonstrated that mRGCs were indeed among the surviving RGCs after axotomy (Robinson & Madison, 2004) and the last study suggested that involvement of the PI3K/Akt pathway was key to their survival (Li et al., 2008). Other evidences of mRGCs robustness are provided by studies on cell toxicity to monosodium glutamate (Chambille & Serviere 1993; Hannibal, Vrang, Card, & Fahrenkrug, 2001). The neuroprotective role of pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide specifically expressed by mRGCs, has been emphasized by studies on experimental models of monosodium glutamate toxicity and ocular ischemia (Atlasz et al., 2008; Seki et al., 2010). Furthermore, the neuroprotective effect of PACAP administration was also shown in RGCs after optic nerve transection, providing a plausible candidate for mRGCs resistance to death after axotomy (Seki, Itoh, Nakamachi, & Shioda, 2008).

Our results in mitochondrial optic neuropathies make it tempting to speculate that spared mRGCs have specific metabolic properties and probably have high mitochondrial activity. This is also suggested by our preliminary results with immunostaining of mRGCs by mitochondrial and COX antibodies (Carla Giordano, unpublished results). Intense COX staining has also been demonstrated by others in surviving RGCs after axotomy (von Bussmann et al., 1993). The metabolic robustness of mRGCs to mitochondrial dysfunction may be specific to complex I impairment, considering that LHON is due to mitochondrial DNA point mutations affecting complex I subunits, as well as a mouse model of LHON was obtained by intravitreal injection of rotenone, a classic complex I inhibitor (Carelli et al., 2004; Zhang et al., 2006). In the latter, the authors noted the preservation of pupillary light response as in patients with LHON (Zhang et al., 2006). Moreover, a complex I defect has also been documented in OPA1 mutant fibroblasts from DOA patients (Zanna et al., 2008). Complex I dysfunction in mitochondrial optic neuropathies has been shown to lead at least to three pathogenic mechanisms: (1) ATP synthesis driven by complex I-dependent substrates (malate and glutamate) may be impaired even if the complex II-dependent pathway can complement this defect (Carelli et al., 2007). (2) ROS production is chronically increased challenging the antioxidant intra-mitochondrial enzymatic

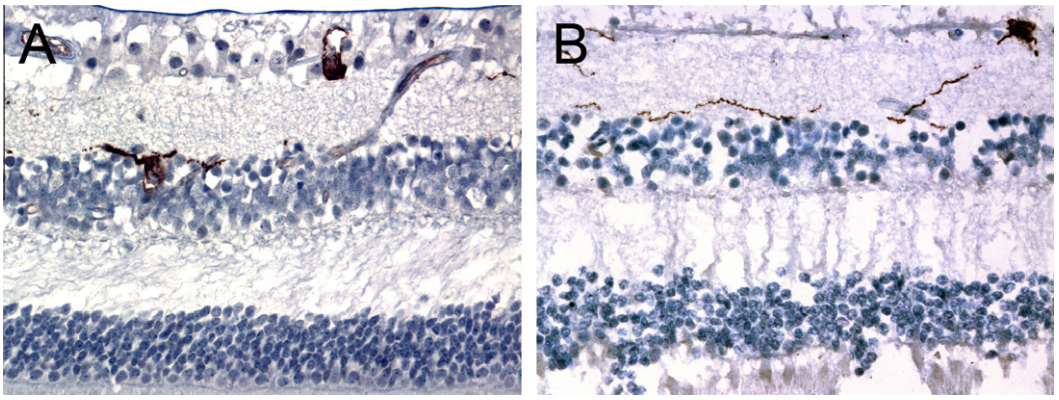


Fig. 2. Postmortem human retinas, immunoperoxidase staining for melanopsin, light microscopy; (A) Retina from a normal 58-year-old male demonstrates melanopsin-positive retinal ganglion cells (mRGCs) in both the RGC layer (RGCL) and the inner nuclear layer (INL). (B) Retina from a 52-year-old LHON male patient carrying the 11778/ND4 mutation of mtDNA with severe optic neuropathy. Note the positive staining for a single mRGC in the RGCL, its dendritic arborization and the absence of non-melanopsin RGCs (as compared to micrograph A). In LHON retinas mRGCs can also be found within the INL at a similar frequency with normal controls (La Morgia et al., 2010). (Both A and B, magnification 400×.)

activities (manganese superoxide dismutase and glutathione peroxidase) (Carelli et al., 2007). (3) Cells are sensitized to undergo apoptosis by shifting the threshold voltage for permeability transition pore (PTP) opening (Porcelli et al., 2009). Thus, the protection of mRGCs in comparison to the other RGCs may be mediated through compensation of any of these mechanisms. Furthermore, melanopsin-driven photoreception has been reported to trigger the mobilization of intracellular calcium and, considering the essential role played by the mitochondrial network in buffering calcium, we can postulate an important and specific role played by mitochondria in the metabolic homeostasis of mRGCs (Hartwick et al., 2007; Kumbalasiri, Rollag, Isoldi, Castrucci, & Provencio, 2007). The 'metabolic robustness' assumed for mRGCs could be derived by a specific resistance to ROS due to handling of light and calcium mobilization capability of these cells. The mRGCs photoreceptive properties stand in support of this hypothesis. Light exposure to normal RGCs may be directly harmful in the setting of dysfunctional mitochondria, as in LHON and DOA, by increasing the ROS-mediated damage (Osborne et al., 2008). We may speculate that mRGCs may be intrinsically protected from light damage and ROS overproduction by expressing the melanopsin photopigment, which absorbs short wavelength light. Thus, melanopsin itself may be the other candidate for a neuroprotective role besides the above mentioned PACAP. At the present stage all these are speculations and working hypothesis needing solid experimental proofs.

4. Glaucoma optic neuropathy: are mRGCs lost or spared?

Glaucoma is the most common optic neuropathy (Quigley & Broman, 2006). The late stages of glaucoma are characterized by an extensive loss of RGCs, which leads to optic atrophy and blindness. Classically, the loss of RGCs in glaucoma follows a physical pattern selecting for nerve fibers in the arcuate bundles first. Recently, the involvement of mRGCs as well as regular RGCs in glaucoma has been postulated (Jean-Louis, Zizi, Lazzaro & Wolintz, 2008). Indeed, there are two studies in animal models of glaucoma that suggest a loss of mRGCs or melanopsin photopigment leading to impairment of circadian rhythms regulation (Drouyer et al., 2008; Wang et al., 2008). Furthermore, preliminary data in humans suggested abnormal circadian rhythm of melatonin secretion and of light-induced melatonin suppression in glaucoma patients (Cooper et al., 2008; Perez Rico, de la Villa, & Blanco, 2010). Interestingly, the pupillary light reflex is also compromised in glaucoma patients, as documented by pupillographic studies (Kalaboukhova, Fridhammar, & Lindblom, 2007). However, others have shown that mRGCs may be spared in some animal models of chronic ocular hypertension (Jakobs, Libby, Ben, John, & Masland, 2005; Li et al., 2006).

Thus, further studies are warranted to clarify the involvement of mRGCs in the natural history of glaucoma, and to investigate the occurrence of sleep and circadian rhythms abnormalities in patients with late-stage glaucoma.

5. Melanopsin genetics and seasonal affective disorders

A circadian misalignment has been demonstrated in many mood disorders (Lewy, 2009). The circadian basis for affective disorders is well supported, especially for winter depression, i.e. seasonal affective disorder (SAD), which is characterized by the annual recurrence of the mood disturbance in fall/winter, with remission in spring/summer. Abnormalities of hormonal circadian rhythms have been reported in SAD and light therapy, particularly with blue light, has been demonstrated as an effective treatment for this disorder (Glickman, Byrne, Pineda, Hauck, & Brainard, 2006; Lewy et al., 2009). These data suggest the involvement of the circadian system and, in particular, of the mechanisms underlying the photoentrainment of circadian rhythms in the pathogenesis of the disease. These findings prompted the investigation of the genetic variation of the *OPN4* gene that encodes for melanopsin, and a missense variant (P10L) was found associated to SAD, suggesting the possible role of the mRGCs-system in its pathogenesis (Roecklein et al., 2009).

6. Melanopsin and photophobia in migraine

Another recently published study reported on the contribution of mRGCs to photophobia in migraine (Noseda et al., 2010). This contribution has been substantiated by the observation that blind people with light perception, despite the severe defective visual acuity, maintain photophobia during migraine attacks, at difference with blind people without light perception (mostly of them with ocular enucleation). The authors proved, using anterograde tracing and single-unit recordings, that the synaptic contact with trigemino-vascular neurons in the posterior thalamus mainly derives from mRGCs, suggesting a major role of the non-image forming system of the retina in mediating migraine-related photophobia (Noseda et al., 2010). The contribution of mRGCs to photophobia has also been documented in rodless and coneless mice (Matynia, Parikh, Chen, Nusinowitz, & Gorin, 2010; Semo et al., 2010).

7. Melanopsin and aging

The aging process is associated with well-documented changes in sleep. These changes may result from an abnormal homeostatic regulation of sleep, expressed as an impaired consolidation of NREM sleep highlighted by a reduction of slow wave activity and increased awakenings during the night (Dijk, Duffy, & Czeisler, 2000), and/or from a dysfunction of circadian rhythms regulation (Cajochen, Münch, Knoblauch, Blatter, & Wirz-Justice, 2006). Decline in the amplitude of circadian rhythms such as core body temperature, melatonin and cortisol secretion have been reported in the elderly (Cajochen et al., 2006). Moreover, a phase-advance of circadian rhythms, in particular of the sleep–wake cycle, and a shortening of the circadian period are also noted in the elderly (Carrier, Paquet, Morettini, & Touchette, 2002). Many factors have been implicated in the occurrence of circadian timing disturbances with age. Among them we mention: (1) Neurodegenerative changes in the SCN, i.e. reduction of vasopressin and vasoactive intestinal peptide expressing neurons. (2) Alterations in the pineal gland, melatonin levels and melatonin receptors with a decline in the amplitude of melatonin rhythm and expression of melatonin receptors (Wu & Swaab, 2007). (3) Reduction of light input to the SCN due to a decreased capacity of the semicataractous lens to transmit short wavelength light (Turner & Mainster, 2008). Furthermore, many degenerative changes occur in the retina and optic nerve with aging (Johnson, Miao, & Sadun, 1987; Chakravarthy, Evans, & Rosenfeld, 2010).

The contribution of the mRGCs-system to the occurrence of the circadian misalignment in aging may be more important than expected. We evaluated post-mortem retinal specimens from controls, comparing two subjects in the mid fifties with one subject over eighty (La Morgia et al., 2010). We found that mRGCs, as well as regular RGCs, are lost with age, as previously reported in animal models and humans (Johnson et al., 1987; Semo, Lupi, Peirson, Butler, & Foster, 2003; Esquiva et al., 2010). We also showed that mRGCs are more concentrated around the fovea and this distribution is maintained with aging despite the general loss of mRGCs

(Dacey et al., 2005; Jusuf, Lee, Hannibal, & Grünert, 2007; La Morgia et al., 2010).

The loss of mRGCs and related impairment of circadian rhythms and sleep in the elderly is at odds with the metabolic robustness of mRGCs documented in mitochondrial optic neuropathies particularly since mitochondrial dysfunction is implicated in the aging process (Trifunovic & Larsson, 2008). This paradox needs to be investigated.

8. Future directions

The role of mRGCs-dependent functions in human pathology is just beginning to be understood. It is already well established that loss of these cells may be implicated in the pathogenesis of circadian, sleep, cognitive and mood disorders.

Glaucoma is a key model that needs to be investigated in regards to circadian photoreception in optic neuropathies. Moreover, retinal dysfunction and RGCs loss has also been documented in several neurodegenerative diseases including Alzheimer and Parkinson disease (Archibald, Clarke, Mosimann, & Burn, 2009; Berisha, Feke, Trempe, McMeel, & Schepens, 2007; Bodis-Wollner, 2009; Hinton, Sadun, Blanks, & Miller, 1986). In both diseases there is evidence of circadian dysfunction (Willis, Kelly, & Kennedy, 2008; Wu & Swaab, 2007) and efficacy of light as therapy (Burns, Allen, Tomenson, Duignan, & Byrne, 2009; Paus et al., 2007). A reasonable link connecting the retinal pathology and circadian dysfunction in Alzheimer and Parkinson disease may be the involvement of mRGCs in the neurodegenerative process.

Preliminary data associating genetic variations of the *OPN4* gene with SAD suggests that polymorphic variants of this gene may also contribute to the human chronotype, i.e. the preference to be active early or late in the day (morningness/eveningness) (Roenneberg et al., 2007). Interestingly, melanopsin is implicated in sleep homeostasis, i.e. build-up of sleep pressure during the wakefulness, as demonstrated in the *Opn4*$^{-/-}$ mouse model, in which a significant attenuation of electroencephalographic slow wave activity, a marker of sleep homeostasis, was documented (Tsai et al., 2009). Similarly, a different sleep homeostatic regulation has been found in morningness/eveningness types, again potentially linking the role of melanopsin in contributing to the chronotype (Mongrain, Carrier, & Dumont, 2006; Mongrain & Dumont, 2007).

It is now well established that many human pathological conditions are responsive to the light stimulation of the circadian pacemaker (SCN) including circadian rhythm sleep disorders, (delayed and advanced sleep phase syndrome, shift work disorder, jet lag and irregular sleep–wake rhythm disorder), age-related sleep disturbances, Alzheimer and Parkinson disease. In all these conditions light therapy may be effective (Burns et al., 2009; Dowling et al., 2008; Morgenthaler et al., 2007; Paus et al., 2007). Better definition of the parameters of the light sources for light therapy trials is warranted to optimize the efficacy of this approach in stimulating the mRGCs-system.

The mounting evidence for a direct contribution of mRGCs to vision may indicate a possible avenue for intervention (Brown, Gigg, Piggins, & Lucas, 2010; Chen, Ecker, & Hattar, 2010; Dacey et al., 2005; Gias, Semo, Vugler, & Coffey 2010). The extent to which mRGCs contribute to visual acuity is not fully defined yet, nor is the contribution of other recently described opsin-expressing cells in the inner retina (Semo, Vugler, & Jeffery, 2007). These more recent observations may have important implications for a subset of blind subjects where mRGCs are spared. This is the case for mitochondrial optic neuropathies, such as LHON and DOA, as we have demonstrated that mRGCs are relatively spared in comparison to regular RGCs. It would be extremely useful to distinguish the visual acuity driven by the mRGC channel as distinct from that provided by rods and cones. Currently the physiological separation of these two pathways has been achieved by a pupillometric protocol intended to elicit the pupillary light response (Park et al., 2010). It has been shown that awareness of light, i.e. a form of blind sight, may be evoked in blind patients, such as those lacking an outer retina, using short-wavelength stimulus, specific for mRGCs (Zaidi et al., 2007). Another promising approach has been illustrated when visual function was partially restored in a mouse model of retinal degeneration by an ectopic expression of melanopsin into regular RGCs (Lin, Koizumi, Tanaka, Panda, & Masland 2008).

The recent recognition that mRGCs project to many structures of the central nervous system contributing to vision, opens the question of their role in many light-mediated responses, such as photophobia in migraine. Similarly, photosensitivity and electroencephalographic photoparoxysmal responses may be modulated by the mRGCs projections.

Our studies on mitochondrial optic neuropathies, LHON and DOA, as well as a few other recent studies on the role of the mRGCs-system in human patho-physiology gives promise that the next decade will deliver us many more surprises in the field of melanopsin.

References

Archibald, N. K., Clarke, M. P., Mosimann, U. P., & Burn, D. J. (2009). The retina in Parkinson's disease. *Brain, 132*(Pt 5), 1128–1145.

Atlasz, T., Szabadfi, K., Kiss, P., Babai, N., Koszegi, Z., Tamas, A., et al. (2008). PACAP-mediated neuroprotection of neurochemically identified cell types in MSG-induced retinal degeneration. *Journal of Molecular Neuroscience, 36*(1–3), 97–104.

Berisha, F., Feke, G. T., Trempe, C. L., McMeel, J. W., & Schepens, C. L. (2007). Retinal abnormalities in early Alzheimer's disease. *Investigative Ophthalmology and Visual Science, 48*(5), 2285–2289.

Berson, D. M., Dunn, F. A., & Takao, M. (2002). Phototransduction by retinal ganglion cells that set the circadian clock. *Science, 295*(5557), 1070–1073.

Berson, D. M. (2003). Strange vision: Ganglion cells as circadian photoreceptors. *Trends in Neurosciences, 26*(6), 314–320.

Bodis-Wollner, I. (2009). Retinopathy in Parkinson disease. *Journal of Neural Transmission, 116*(11), 1493–1501.

Bose, S., Dhillon, N., Ross-Cisneros, F. N., & Carelli, V. (2005). Relative post-mortem sparing of afferent pupil fibers in a patient with 3460 Leber's hereditary optic neuropathy. *Graefes Archive for Clinical and Experimental Ophthalmology, 243*(11), 1175–1179.

Brainard, G. C., Hanifin, J. P., Greeson, J. M., Byrne, B., Glickman, G., Gerner, E., et al. (2001). Action spectrum for melatonin regulation in humans: Evidence for a novel circadian photoreceptor. *Journal of Neuroscience, 21*(16), 6405–6412.

Bremner, F. D., Tomlin, E. A., Shallo-Hoffmann, J., Votruba, M., & Smith, S. E. (2001). The pupil in dominant optic atrophy. *Investigative Ophthalmology and Visual Science, 42*(3), 675–678.

Brown, T. M., Gigg, J., Piggins, H. D., & Lucas, R. J. (2010). Melanopsin-dependent activation of dorsal lateral geniculate neurons. ARVO 2010, poster no. 672/D736.

Burns, A., Allen, H., Tomenson, B., Duignan, D., & Byrne, J. (2009). Bright light therapy for agitation in dementia: A randomized controlled trial. *International Psychogeriatrics, 21*(4), 711–721.

Cajochen, C., Münch, M., Knoblauch, V., Blatter, K., & Wirz-Justice, A. (2006). Age-related changes in the circadian and homeostatic regulation of human sleep. *Chronobiology International, 23*(1–2), 461–474.

Carelli, V., La Morgia, C., Iommarini, L., Carroccia, R., Mattiazzi, M., Sangiorgi, S., et al. (2007). Mitochondrial optic neuropathies: How two genomes may kill the same cell type? *Bioscience Reports, 27*(1–3), 173–184.

Carelli, V., Ross-Cisneros, F. N., & Sadun, A. A. (2004). Mitochondrial dysfunction as a cause of optic neuropathies. *Progress in Retinal and Eye Research, 23*, 53–89.

Carrier, J., Paquet, J., Morettini, J., & Touchette, E. (2002). Phase advance of sleep and temperature circadian rhythms in the middle years of life in humans. *Neuroscience Letters, 320*(1–2), 1–4.

Chakravarthy, U., Evans, J., & Rosenfeld, P. J. (2010). Age related macular degeneration. *BMJ, 340*, c981.

Chambille, I., & Serviere, J. (1993). Neurotoxic effects of neonatal injections of monosodium L-glutamate (L-MSG) on the retinal ganglion cell layer of the golden hamster: Anatomical and functional consequences on the circadian system. *Journal of Comparative Neurology, 338*, 67–82.

Chen, S. -K., Ecker, J. L., & Hattar, S. (2010). Understanding the complexity of ipRGCs targeting and functions. ARVO meeting, poster no. 664/D728.

Cooper, H. M., Herbin, M., & Nevo, E. (1993). Ocular regression conceals adaptive progression of the visual system in a blind subterranean mammal. *Nature, 361*(6408), 156–159.

Cooper, H. M., Drouyer, E., Dkhissi-Benyahya, O., Gronfier, C., Chiquet, C., WoldeMussie, E., et al. (2008). Effects of glaucoma on the circadian timing system in mice and men. ARVO meeting, poster no. 182/A327.

Czeisler, C. A., Shanahan, T. L., Klerman, E. B., Martens, H., Brotman, D. J., Emens, J. S., et al. (1995). Suppression of melatonin secretion in some blind patients by exposure to bright light. *New England Journal of Medicine, 332*(1), 6–11.

Dacey, D. M., Liao, H. W., Peterson, B. B., Robinson, F. R., Smith, V. C., Pokorny, J., et al. (2005). Melanopsin-expressing ganglion cells in primate retina signal colour and irradiance and project to the LGN. *Nature, 433*(7027), 749–754.

David-Gray, Z. K., Janssen, J. W., DeGrip, W. J., Nevo, E., & Foster, R. G. (1998). Light detection in a 'blind' mammal. *Nature Neuroscience, 1*(8), 655–656.

Dijk, D. J., Duffy, J. F., & Czeisler, C. A. (2000). Contribution of circadian physiology and sleep homeostasis to age-related changes in human sleep. *Chronobiology International, 17*(3), 285–311.

Dijk, D. J., & Archer, S. N. (2009). Light, sleep, and circadian rhythms: Together again. *PLoS Biology, 7*(6), e1000145.

Dowling, G. A., Burr, R. L., Van Someren, E. J., Hubbard, E. M., Luxenberg, J. S., Mastick, J., et al. (2008). Melatonin and bright-light treatment for rest-activity disruption in institutionalized patients with Alzheimer's disease. *Journal of the American Geriatrics Society, 56*(2), 239–246.

Drouyer, E., Dkhissi-Benyahya, O., Chiquet, C., WoldeMussie, E., Ruiz, G., Wheeler, L. A., et al. (2008). Glaucoma alters the circadian timing system. *PLoS ONE, 3*(12), e3931.

Esquiva, G., Gonzalez-Mendez, I., Fernandez-Sanchez, L., Garcia-Martin, E., Pinilla, I., Garcia-Fernandez, J., et al. (2010). Degeneration of melanopsin photosensitive retinal ganglion cells in human retinas with aging and in animal models of retinitis pigmentosa. ARVO meeting, poster no. 680/D744.

Foster, R. G., Provencio, I., Hudson, D., Fiske, S., De Grip, W., & Menaker, M. (1991). Circadian photoreception in the retinally degenerate mouse (rd/rd). *Journal of Comparative Physiology A, 169*(1), 39–50.

Freedman, M. S., Lucas, R. J., Soni, B., von Schantz, M., Muñoz, M., David-Gray, Z., et al. (1999). Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors. *Science, 284*(5413), 502–504.

Fu, Y., Zhong, H., Wang, M. H., Luo, D. G., Liao, H. W., Maeda, H., et al. (2005). Intrinsically photosensitive retinal ganglion cells detect light with a vitamin A-based photopigment, melanopsin. *Proceedings of the National Academy of Sciences of the United States of America, 102*(29), 10339–11044.

Gias, C., Semo, M., Vugler, A. A., & Coffey, P. J. (2010). Melanopsin cortical response in the rodless coneless mouse to light stimulation revealed using optical imaging of intrinsic signals. ARVO meeting, poster no. 673/D737.

Glickman, G., Byrne, B., Pineda, C., Hauck, W. W., & Brainard, G. C. (2006). Light therapy for seasonal affective disorder with blue narrow-band light-emitting diodes (LEDs). *Biological Psychiatry, 59*(6), 502–507.

Guler, A. D., Ecker, J. L., Lall, G. S., Haq, S., Altimus, C. M., Liao, H. W., et al. (2008). Melanopsin cells are the principal conduits for rod–cone input to non-image-forming vision. *Nature, 453*, 102–105.

Hannibal, J., Vrang, N., Card, J. P., & Fahrenkrug, J. (2001). Light-dependent induction of cFos during subjective day and night in PACAP-containing ganglion cells of the retinohypothalamic tract. *Journal of Biological Rhythms, 16*, 457–470.

Hannibal, J., Hindersson, P., Knudsen, S. M., Georg, B., & Fahrenkrug, J. (2002). The photopigment melanopsin is exclusively present in PACAP containing retinal ganglion cells of the retinohypothalamic tract. *Journal of Neuroscience, 22*(RC191), 1–7.

Hannibal, J., Hindersson, P., Nevo, E., & Fahrenkrug, J. (2002). The circadian photopigment melanopsin is expressed in the blind subterranean mole rat, Spalax. *NeuroReport, 13*(11), 1411–1414.

Hannibal, J., Hindersson, P., Ostergaard, J., Georg, B., Heegaard, S., Larsen, P. J., et al. (2004). Melanopsin is expressed in PACAP-containing retinal ganglion cells of the human retinohypothalamic tract. *Investigative Ophthalmology and Visual Science, 45*(11), 4202–4209.

Hannibal, J., & Fahrenkrug, J. (2004). Melanopsin containing retinal ganglion cells are light responsive from birth. *Neuroreport, 15*(15), 2317–2320.

Hannibal, J., & Fahrenkrug, J. (2006). Neuronal input pathways to the brain's biological clock and their functional significance. *Advances in Anatomy, Embryology and Cell Biology, 182*, 1–71.

Hartwick, A. T., Bramley, J. R., Yu, J., Stevens, K. T., Allen, C. N., Baldridge, W. H., et al. (2007). Light-evoked calcium responses of isolated melanopsin-expressing retinal ganglion cells. *Journal of Neuroscience, 27*(49), 13468–13480.

Hätönen, T., Laakso, M. L., Heiskala, H., Alila-Johansson, A., Sainio, K., & Santavuori, P. (1998). Bright light suppresses melatonin in blind patients with neuronal ceroid-lipofuscinoses. *Neurology, 50*(5), 1445–1450.

Hatori, M., Le, H., Vollmers, C., Keding, S. R., Tanaka, N., Schmedt, C., et al. (2008). Inducible ablation of melanopsin-expressing retinal ganglion cells reveals their central role in non-image forming visual responses. *PLoS ONE, 3*, e2451.

Hattar, S., Liao, H. W., Takao, M., Berson, D. M., & Yau, K. W. (2002). Melanopsin-containing retinal ganglion cells: Architecture, projections, and intrinsic photosensitivity. *Science, 295*(5557), 1065–1070.

Hattar, S., Kumar, M., Park, A., Tong, P., Tung, J., Yau, K. W., et al. (2006). Central projections of melanopsin-expressing retinal ganglion cells in the mouse. *Journal of Comparative Neurology, 497*(3), 326–349.

Hendrickson, A. E., Wagoner, N., & Cowan, W. M. (1972). An autoradiographic and electron microscopic study of retino-hypothalamic connections. *Zeitschrift für Zellforschung und Mikroskopische Anatomie, 135*, 1–26.

Hinton, D. R., Sadun, A. A., Blanks, J. C., & Miller, C. A. (1986). Optic-nerve degeneration in Alzheimer's disease. *New England Journal of Medicine, 315*(8), 485–487.

Jakobs, T. C., Libby, R. T., Ben, Y., John, S. W., & Masland, R. H. (2005). Retinal ganglion cell degeneration is topological but not cell type specific in DBA/2J mice. *Journal of Cell Biology, 171*(2), 313–325.

Jean-Louis, G., Zizi, F., Lazzaro, D. R., & Wolintz, A. H. (2008). Circadian rhythm dysfunction in glaucoma: A hypothesis. *J Circadian Rhythms, 10*(6), 1.

Johnson, B. M., Miao, M., & Sadun, A. A. (1987). Age-related decline of human optic nerve axon populations. *Age, 10*, 5–9.

Jusuf, P. R., Lee, S. C., Hannibal, J., & Grünert, U. (2007). Characterization and synaptic connectivity of melanopsin-containing ganglion cells in the primate retina. *European Journal of Neuroscience, 26*(10), 2906–2921.

Kalaboukhova, L., Fridhammar, V., & Lindblom, B. (2007). Relative afferent pupillary defect in glaucoma: A pupillometric study. *Acta Ophthalmologica Scandinavica, 85*(5), 519–525.

Kawasaki, A., Herbst, K., Sander, B., & Milea, D. (2010). Selective wavelength pupillometry in Leber hereditary optic neuropathy. *Clinical and Experimental Ophthalmology, 38*(3), 322–324.

Kumbalasiri, T., Rollag, M. D., Isoldi, M. C., Castrucci, A. M., & Provencio, I. (2007). Melanopsin triggers the release of internal calcium stores in response to light. *Photochemistry and Photobiology, 83*(2), 273–279.

La Morgia, C., Ross-Cisneros, F. N., Sadun, A. A., Hannibal, J., Munarini, A., Mantovani, V., et al. (2010). Melanopsin retinal ganglion cells are resistant to neurodegeneration in mitochondrial optic neuropathies. *Brain, 133*, 2426–2438.

Lewy, A. J. (2009). Circadian misalignment in mood disturbances. *Current Psychiatry Reports, 11*(6), 459–465.

Lewy, A. J., Emens, J. S., Songer, J. B., Sims, N., Laurie, A. L., Fiala, S. C., et al. (2009). Winter depression: Integrating mood, circadian rhythms, and the sleep/wake and light/dark cycles into a bio-psycho-social–environmental model. *Sleep Medicine Clinics, 4*(2), 285–299.

Li, R. S., Chen, B. Y., Tay, D. K., Chan, H. H., Pu, M. L., & So, K. F. (2006). Melanopsin-expressing retinal ganglion cells are more injury-resistant in a chronic ocular hypertension model. *Investigative Ophthalmology and Visual Science, 47*(7), 2951–2958.

Li, S. Y., Yau, S. Y., Chen, B. Y., Tay, D. K., Lee, V. W., Pu, M. L., et al. (2008). Enhanced survival of melanopsin-expressing retinal ganglion cells after injury is associated with the PI3 K/Akt pathway. *Cellular and Molecular Neurobiology, 28*(8), 1095–1107.

Lin, B., Koizumi, A., Tanaka, N., Panda, S., & Masland, R. H. (2008). Restoration of visual function in retinal degeneration mice by ectopic expression of melanopsin. *Proceedings of the National Academy of Sciences of the United States of America, 105*(41), 16009–16014.

Lucas, R. J., Freedman, M. S., Muñoz, M., Garcia-Fernández, J. M., & Foster, R. G. (1999). Regulation of the mammalian pineal by non-rod, non-cone, ocular photoreceptors. *Science, 284*(5413), 505–507.

Lucas, R. J., Hattar, S., Takao, M., Berson, D. M., Foster, R. G., & Yau, K. W. (2003). Diminished pupillary light reflex at high irradiances in melanopsin-knockout mice. *Science, 299*, 245–247.

Lupi, D., Oster, H., Thompson, S., & Foster, R. G. (2008). The acute light-induction of sleep is mediated by OPN4-based photoreception. *Nature Neuroscience, 11*(9), 1068–1073.

Matynia, A., Parikh, S., Chen, B., Nusinowitz, S., & Gorin, M. B. (2010). Behavioral and pharmacological analysis of light associated allodynia. ARVO meeting, poster no. 675/D739.

Mongrain, V., Carrier, J., & Dumont, M. (2006). Circadian and homeostatic sleep regulation in morningness–eveningness. *Journal of Sleep Research, 15*(2), 162–166.

Mongrain, V., & Dumont, M. (2007). Increased homeostatic response to behavioral sleep fragmentation in morning types compared to evening types. *Sleep, 30*(6), 773–780.

Moore, R. Y., & Lenn, N. J. (1972). A retinohypothalamic projection in the rat. *Journal of Comparative Neurology, 146*, 1–14.

Morgenthaler, T. I., Lee-Chiong, T., Alessi, C., Friedman, L., Aurora, R. N., Boehlecke, B., et al. (2007). Practice parameters for the clinical evaluation and treatment of circadian rhythm sleep disorders. An American academy of sleep medicine report. Standards of practice committee of the American academy of sleep medicine. *Sleep, 30*(11), 1445–1459.

Mure, L. S., Rieux, C., Hattar, S., & Cooper, H. M. (2007). Melanopsin-dependent nonvisual responses: Evidence for photopigment bistability in vivo. *Journal of Biological Rhythms, 22*(5), 411–424.

Noseda, R., Kainz, V., Jakubowski, M., Gooley, J. J., Saper, C. B., Digre, K., et al. (2010). A neural mechanism for exacerbation of headache by light. *Nature Neuroscience, 13*(2), 239–245.

Osborne, N. N., Li, G. Y., Ji, D., Mortiboys, H. J., & Jackson, S. (2008). Light affects mitochondria to cause apoptosis to cultured cells: Possible relevance to ganglion cell death in certain optic neuropathies. *Journal of Neurochemistry, 105*, 2013–2028.

Park, J. C., Moura, A. L., Raza, A. S., Palmer, N., Gavazi, E., Tsang, S. H., et al. (2010). Toward a clinical protocol for assessing rod, cone and melanopsin contributions to the human pupil response. ARVO meeting, poster no. 1453/A602.

Paus, S., Schmitz-Hübsch, T., Wüllner, U., Vogel, A., Klockgether, T., & Abele, M. (2007). Bright light therapy in Parkinson's disease: A pilot study. *Movement Disorders, 22*(10), 1495–1498.

Peirson, S. N., Halford, S., & Foster, R. G. (2009). The evolution of irradiance detection: Melanopsin and the non-visual opsins. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 364*(1531), 2849–2865.

Pérez-Rico, C., de la Villa, P., Blanco, R., Germain, F., Paz-Moreno, J., & Arribas-Gómez, I. (2009). Alterations in nocturnal melatonin secretion in patients with optic neuropathies. *Archivos de la Sociedad Espanola de Oftalmologia, 84*(5), 251–257.

Perez Rico, C., de la Villa, P., & Blanco, R. (2010). Multifocal erg and light-induced melatonin suppression evaluation of the functional integrity of the retinohypothalamic tract in advanced glaucoma. ARVO 2010, poster no. 5490/A545.

Porcelli, A. M., Angelin, A., Ghelli, A., Mariani, E., Martinuzzi, A., Carelli, V., et al. (2009). Respiratory complex I dysfunction due to mitochondrial DNA mutations shifts the voltage threshold for opening of the permeability transition pore toward resting levels. *Journal of Biological Chemistry, 284*(4), 2045–2052.

Provencio, I., Rodriguez, I. R., Jiang, G., Hayes, W. P., Moreira, E. F., & Rollag, M. D. (2000). A novel human opsin in the inner retina. *Journal of Neuroscience, 20*(2), 600–605.

Provencio, I., Rollag, M. D., & Castrucci, A. M. (2002). Photoreceptive net in the mammalian retina. This mesh of cells may explain how some blind mice can still tell day from night. *Nature, 415*(6871), 493.

Quigley, H. A., & Broman, A. T. (2006). The number of people with glaucoma worldwide in 2010 and 2020. *British Journal of Ophthalmology, 90*(3), 262–267.

Reppert, S. M., & Weaver, D. R. (2002). Coordination of circadian timing in mammals. *Nature, 418*(6901), 935–941.

Robinson, G. A., & Madison, R. D. (2004). Axotomized mouse retinal ganglion cells containing melanopsin show enhanced survival, but not enhanced axon regrowth into a peripheral nerve graft. *Vision Research, 44*(23), 2667–2674.

Roecklein, K. A., Rohan, K. J., Duncan, W. C., Rollag, M. D., Rosenthal, N. E., Lipsky, R. H., et al. (2009). A missense variant (P10L) of the melanopsin (OPN4) gene in seasonal affective disorder. *Journal of Affective Disorders, 114*(1–3), 279–285.

Roenneberg, T., Kuehnle, T., Juda, M., Kantermann, T., Allebrandt, K., Gordijn, M., et al. (2007). Epidemiology of the human circadian clock. *Sleep Medicine Reviews, 11*(6), 429–438.

Sack, R. L., & Lewy, A. J. (2001). Circadian rhythm sleep disorders: Lessons from the blind. *Sleep Medicine Reviews, 5*(3), 189–206.

Sadun, A. A., Schaechter, J. D., & Smith, L. E. (1984). A retinohypothalamic pathway in man: Light mediation of circadian rhythms. *Brain Research, 302*(2), 371–377.

Sadun, A. A., Win, P. H., Ross-Cisneros, F. N., Walker, S. O., & Carelli, V. (2000). Leber's hereditary optic neuropathy differentially affects smaller axons in the optic nerve. *Transactions of the American Ophthalmological Society, 98*, 223–232.

Saper, C. B., Lu, J., Chou, T. C., & Gooley, J. (2005). The hypothalamic integrator for circadian rhythms. *Trends in Neurosciences, 28*, 152–157.

Seki, T., Itoh, H., Nakamachi, T., & Shioda, S. (2008). Suppression of ganglion cell death by PACAP following optic nerve transection in the rat. *Journal of Molecular Neuroscience, 36*(1–3), 57–60.

Seki, T., Itoh, H., Nakamachi, T., Endo, K., Wada, Y., Nakamura, K., et al. (2010). Suppression of rat retinal ganglion cell death by PACAP following transient ischemia induced by high intraocular pressure. *Journal of Molecular Neuroscience*(June 29) (Epub ahead of print).

Semo, M., Lupi, D., Peirson, S. N., Butler, J. N., & Foster, R. G. (2003). Light-induced c-fos in melanopsin retinal ganglion cells of young and aged rodless/coneless (rd/rd cl) mice. *European Journal of Neuroscience, 18*(11), 3007–3017.

Semo, M., Vugler, A. A., & Jeffery, G. (2007). Paradoxical opsin expressing cells in the inner retina that are augmented following retinal degeneration. *European Journal of Neuroscience, 25*(8), 2296–2306.

Semo, M., Gias, C., Lawrence, J. M., Ahmado, A., Coffey, P. J., & Vugler, A. A. (2010). Melanopsin mediated photophobia in adult rodless and coneless mice. ARVO 2010, poster no. 674/D738.

Skene, D. J., & Arendt, J. (2007). Circadian rhythm sleep disorders in the blind and their treatment with melatonin. *Sleep Medicine, 8*(6), 651–655.

Tarttelin, E. E., Bellingham, J., Bibb, L. C., Foster, R. G., Hankins, M. W., Gregory-Evans, K., et al. (2003). Expression of opsin genes early in ocular development of humans and mice. *Experimental Eye Research, 76*(3), 393–396.

Thapan, K., Arendt, J., & Skene, D. J. (2001). An action spectrum for melatonin suppression: Evidence for a novel non-rod, non-cone photoreceptor system in humans. *Journal of Physiology, 535*(Pt. 1), 261–267.

Trifunovic, A., & Larsson, N. G. (2008). Mitochondrial dysfunction as a cause of ageing. *Journal of Internal Medicine, 263*(2), 167–178.

Tsai, J. W., Hannibal, J., Hagiwara, G., Colas, D., Ruppert, E., Ruby, N. F., et al. (2009). Melanopsin as a sleep modulator: Circadian gating of the direct effects of light on sleep and altered sleep homeostasis in Opn4(−/−) mice. *PLoS Biology, 7*(6), e1000125.

Tu, D. C., Zhang, D., Demas, J., Slutsky, E. B., Provencio, I., Holy, T. E., et al. (2005). Physiologic diversity and development of intrinsically photosensitive retinal ganglion cells. *Neuron, 48*(6), 987–999.

Turner, P. L., & Mainster, M. A. (2008). Circadian photoreception: Ageing and the eye's important role in systemic health. *British Journal of Ophthalmology, 92*(11), 1439–1444.

Vandewalle, G., Maquet, P., & Dijk, D. J. (2009). Light as a modulator of cognitive brain function. *Trends in Cognitive Sciences, 13*(10), 429–438.

Vandewalle, G., Schmidt, C., Albouy, G., Sterpenich, V., Darsaud, A., Rauchs, G., et al. (2007a). Brain responses to violet, blue, and green monochromatic light exposures in humans: Prominent role of blue light and the brainstem. *PLoS One, 2*(11), e1247.

Vandewalle, G., Gais, S., Schabus, M., Balteau, E., Carrier, J., Darsaud, A., et al. (2007b). Wavelength-dependent modulation of brain responses to a working memory task by daytime light exposure. *Cerebral Cortex, 17*(12), 2788–2795.

von Bussmann, K. A., Garey, L. J., & Jen, L. S. (1993). Injury-resistant retinal ganglion cells that are rich in cytochrome oxidase. *NeuroReport, 4*(3), 247–250.

Vugler, A. A., Redgrave, P., Semo, M., Lawrence, J., Greenwood, J., & Coffey, P. J. (2007). Dopamine neurones form a discrete plexus with melanopsin cells in normal and degenerating retina. *Experimental Neurology, 205*(1), 26–35.

Vugler, A. A., Semo, M., Joseph, A., & Jeffery, G. (2008). Survival and remodeling of melanopsin cells during retinal dystrophy. *Visual Neuroscience, 25*(2), 125–138.

Wakakura, M., & Yokoe, J. (1995). Evidence for preserved direct pupillary light response in Leber's hereditary optic neuropathy. *British Journal of Ophthalmology, 79*(5), 442–446.

Wang, H. Z., Lu, Q. J., Wang, N. L., Liu, H., Zhang, L., & Zhan, G. L. (2008). Loss of melanopsin-containing retinal ganglion cells in a rat glaucoma model. *Chinese Medical Journal (English Edition), 121*(11), 1015–1019.

Wee, R., & Van Gelder, R. N. (2004). Sleep disturbances in young subjects with visual dysfunction. *Ophthalmology, 111*(2), 297–302.

Willis, G. L., Kelly, A. M., & Kennedy, G. A. (2008). Compromised circadian function in Parkinson's disease: Enucleation augments disease severity in the unilateral model. *Behavioural Brain Research, 193*(1), 37–47.

Wu, Y. H., & Swaab, D. F. (2007). Disturbance and strategies for reactivation of the circadian rhythm system in aging and Alzheimer's disease. *Sleep Medicine, 8*(6), 623–636.

Zaidi, F. H., Hull, J. T., Peirson, S. N., Wulff, K., Aeschbach, D., Gooley, J. J., et al. (2007). Short-wavelength light sensitivity of circadian, pupillary, and visual awareness in humans lacking an outer retina. *Current Biology, 17*(24), 2122–2128.

Zanna, C., Ghelli, A., Porcelli, A. M., Karbowski, M., Youle, R. J., Schimpf, S., et al. (2008). OPA1 mutations associated with dominant optic atrophy impair oxidative phosphorylation and mitochondrial fusion. *Brain, 131*(Pt. 2), 352–367.

Zhang, X., Jones, D., & Gonzalez-Lima, F. (2006). Neurodegeneration produced by rotenone in the mouse retina: A potential model to investigate environmental pesticide contributions to neurodegenerative diseases. *Journal of Toxicology and Environmental Health, Part A, 69*(18), 1681–1697.

ANNEX 3

European Journal of Neurology 2012 doi:10.1111/j.1468-1331.2012.03701.x

SHORT COMMUNICATION

Loss of temporal retinal nerve fibers in Parkinson disease: a mitochondrial pattern?

C. La Morgia[a,b], P. Barboni[b], G. Rizzo[a,b], M. Carbonelli[c], G. Savini[c], C. Scaglione[a,b], S. Capellari[a,b], S. Bonazza[a,b], M. P. Giannoccaro[a,b], G. Calandra-Buonaura[a,b], R. Liguori[a,b], P. Cortelli[a,b], P. Martinelli[a,b], A. Baruzzi[a,b] and V. Carelli[a,b]

[a]*IRCCS Istituto delle Scienze Neurologiche di Bologna, Bologna;* [b]*Department of Neurological Sciences, University of Bologna, Bologna; and* [c]*IRCCS Fondazione G.B. Bietti, Roma, Italy*

Keywords:
mitochondrial disorders, optic nerve, optical coherence tomography, Parkinson's disease, retina

Received 22 December 2011
Accepted 13 February 2012

Background: Optic nerve involvement is frequent in mitochondrial disease, and retinal abnormalities are described in Parkinson's disease (PD).
Methods: We evaluated retinal nerve fiber layer (RNFL) thickness by optical coherence tomography in 43 patients with PD and in 86 age-matched controls. We considered separately the eyes ipsilateral and contralateral to the most affected body side in patients with PD. ANCOVA analysis, Pearson test, and multiple regression analysis were used ($P < 0.05$).
Results: Patients with PD showed significantly thinner temporal RNFL thickness compared to controls ($P = 0.004$), more evident in the eye contralateral to the most affected body side. Average RNFL thickness significantly correlated with age in both controls and patients with PD (p values ranging from 0.001 to 0.019), whereas in patients with PD RNFL thickness did not correlate with clinical variables.
Conclusions: Our study reveals a loss of retinal nerve fibers in the temporal quadrant in PD, which is typically susceptible in mitochondrial optic neuropathies.

Introduction

Parkinson disease (PD) is one of the most common age-dependent neurodegenerative disorders and is characterized by the loss of dopaminergic neurons in the substantia nigra [1]. The typical clinical picture of PD includes the presence of motor symptoms such as bradykinesia, tremor, and rigidity. However, non-motor symptoms are increasingly recognized in PD, implying wider neurodegenerative involvement [2].

There is mounting evidence of mitochondrial dysfunction in the pathogenesis of PD. Complex I deficiency leads to Parkinsonian features in humans and animal models [3]. Furthermore, monogenic forms of PD are associated with mutations affecting proteins that are either located in mitochondria or may translocate to mitochondria under specific circumstances, such as for the PINK1/Parkin pathway [4].

Correspondence: C. La Morgia, IRCCS Istituto delle Scienze Neurologiche di Bologna, Department of Neurological Sciences, University of Bologna, Italy (tel.: +390512092747; fax: 390512092751; e-mail: chiaralamorgia@gmail.com).
Funding sources: Fondazione G. Galletti.

Mitochondrial optic neuropathies, such as Leber's hereditary optic neuropathy (LHON) and dominant optic atrophy (DOA), are associated with complex I defect and a preferential involvement of the papillo-macular bundle, which enters the optic disk on the infero-temporal side [5–7]. Ocular involvement in PD has been recognized for a long time [8]. More recently, the availability of optical coherence tomography (OCT), which allows objective anatomical measurements in the retina, including the retinal nerve fiber layer (RNFL) thickness, prompted the investigation of retinal ganglion cells and optic nerve in PD [9–14]. In this study, we measured by OCT the RNFL thickness in patients with PD compared with age-matched controls to investigate optic nerve in PD.

Patients and methods

Consecutive patients with PD followed at the IRCCS Istituto delle Scienze Neurologiche di Bologna, were recruited from September 2010 to June 2011. Diagnosis of probable PD was established according to the Gelb criteria [15].

EUROPEAN JOURNAL OF NEUROLOGY
THE OFFICIAL JOURNAL OF THE EUROPEAN FEDERATION OF NEUROLOGICAL SOCIETIES

All study participants underwent a standard ophthalmologic evaluation to exclude spherical or cylindrical refractive errors higher than three or two diopters, respectively, posterior pole pathology such as macular degeneration, and optic neuropathies including glaucoma. Furthermore, patients with unsustained fixation owing to rest tremor were excluded. All participants gave their informed consent to the study, which was approved by the Internal Review Board (Comitato Etico Indipendente, Azienda Unità Sanitaria Locale di Bologna, Protocol #656/CE).

We included 43 patients with PD (65.6 ± 8.4 years; range 46–83) and 86 age-matched controls (mean age 65.5 ± 10.7 years; range 46–85 years). Amongst patients with PD, three had a molecular diagnosis (two carried the G2019S LRRK2 mutation and one a PARK2 exon 2 deletion) and five had first-degree relatives affected without molecular definition. We considered all these eight patients with PD as genetic cases. For all patients, we retrieved disease duration, UPDRS-III (off) score, and stage of disease (Hoehn and Yahr). All subjects underwent RNFL thickness measurement by OCT (StratusOCT, software version 4.0.1; Carl Zeiss Meditec Inc, Dublin, CA, USA). We used the RNFL thickness 3.4 acquisition protocol, as previously described [6,7]. An experienced operator (CLM) performed the examination with pupil dilated. The OCT lenses were adjusted for the patient's refractive error. Polarization was optimized to maximize the reflective signal, and the best centration of the scan with respect to the optic disk was used.

For the statistical analysis, one eye for each control was randomly chosen, having verified that there was no statistical difference between right and left eye [6]. Based on the characteristic asymmetry of PD, we analyzed both the eye ipsilateral and contralateral to the most affected body side in patients with PD, as defined by neurological examination and UPDRS-III score.

Statistical analysis was performed using the SPSS 18.0 package (PASW Statistics 18.0; SPSS Inc., Chicago, IL, USA). Normal distribution of RNFL thickness measurements was evaluated by Kolmogorov test. Differences between patients with PD and controls were assessed by ANCOVA using age and gender as covariates, followed by *post hoc* pairwise comparisons. Significance was assumed for $P < 0.05$ corrected for multiple comparisons according to the Bonferroni method. Correlation bivariate analysis was performed using Pearson coefficient ($P < 0.05$). Multivariate analysis was performed to investigate the effect of genetic, demographic (age and gender), and clinical parameters (age at onset, disease duration, UPDRS-III score, and stage of disease) on RNFL measurements using a multiple regression with a backward stepwise method to obtain a significant model in which all included variables had a $P < 0.05$.

Results

All patients with PD included were on dopaminergic therapy. Disease duration was 9.1 ± 6.5 years, Hoehn and Yahr stage of disease was 2.2 ± 0.9, and UPDRS score was 25.9 ± 12.4. Demographic and the main clinical findings are summarized in Table 1. None of the patients with PD had visual complaints. Five PD eyes (two ipsilateral and three contralateral to the most affected body side) were excluded based on established criteria.

The average RNFL thickness was reduced in patients with PD compared to controls, being close to significance ($P = 0.057$) (Table 1, Fig. 1). Considering separately the four quadrants, only the temporal RNFL thickness was significantly reduced in PD compared to controls ($P = 0.004$) (Table 1). *post hoc* pairwise comparisons showed that the temporal RNFL thickness was significantly reduced for both the contralateral ($P = 0.008$) and the ipsilateral ($P = 0.04$) eye in PD compared to controls (Fig. 1). The significance of RNFL thickness reduction in the temporal quadrant was maintained for the sporadic cases ($P = 0.006$) and genetic cases ($P = 0.039$) considered separately.

Pearson test and multiple regression analysis did not disclose any correlation between genetic, demographic or clinical parameters and RNFL measurements, except for age. Average RNFL thickness correlated with age in both controls ($P = 0.001$; $r = -0.341$) and patients with PD ($P = 0.007$; $r = -0.412$ and $P = 0.019$; $r = -0.371$ for the eye ipsilateral and contralateral to the most affected body side, respectively).

Discussion

Our study reveals a subclinical optic neuropathy, as evidenced by the reduction in the average RNFL thickness, with significant involvement of the nerve fibers entering the temporal quadrant of the optic disk in the overall group of patients with PD. The contralateral eye was more affected than the ipsilateral to the most affected body side, supporting an asymmetry of the neurodegenerative process that involves both the substantia nigra and the eye on the same side. Similarly, an asymmetric impairment has been reported for the olfactory function, further supporting a general asymmetry in the neuropathological process in PD [16]. The reduction in the RNFL thickness was correlated with age in both controls and patients with PD. Overall, these findings add to the mounting evidence of non-motor involvement in PD [2].

Table 1 Summary of demographic and clinical data in Parkinson's disease (PD) and controls

	PD ipsilateral eye (N = 41)	PD contralateral Eye (N = 40)	Controls (N = 86)	ANCOVA
Gender	24 M; 19F		34M; 52F	
Age	65.6 ± 8.4		65.5 ± 10.74	
Disease duration	9.1 ± 6.5			
UPDRS (Off)	25.9 ± 12.4			
HY stage	2.21 ± 0.9			
RNFLavg (μm)	92.2 ± 12.8	91.9 ± 12.1	97 ± 12.4	P = 0.057
RNFL-T (μm)	62.3 ± 10.4	60.9 ± 12.8	68.5 ± 12.3	P = 0.004[a]
RNFL-S (μm)	115.2 ± 17.4	112.4 ± 15.6	118.9 ± 17.4	P = 0.179
RNFL-N (μm)	72.2 ± 18.3	72.9 ± 17.7	76.5 ± 15.7	P = 0.362
RNFL-I (μm)	119.3 ± 19.9	121.5 ± 18.6	124.1 ± 18.6	P = 0.426

[a]*Post hoc* pairwise comparisons: controls vs. PD ipsilateral eye (P = 0.04); controls vs. PD contralateral eye (P = 0.008). Demographic and clinical data of PD and controls and ANCOVA (age and gender as covariates) results are reported.

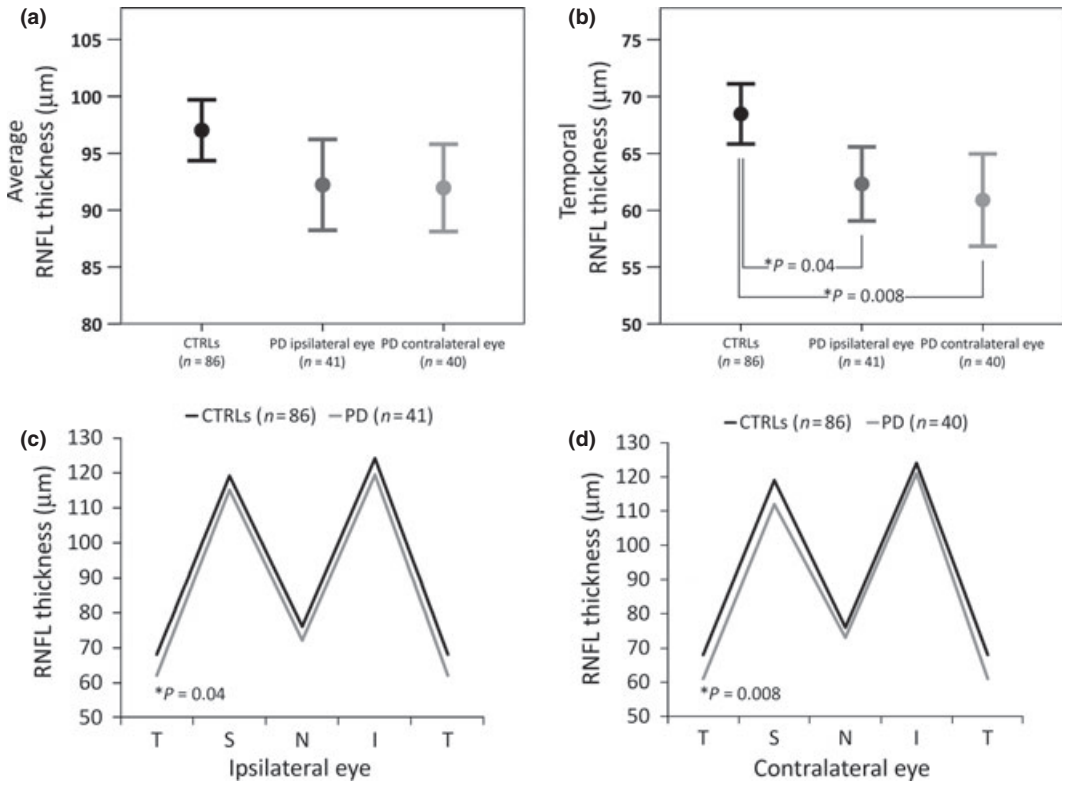


Figure 1 RNFL thickness results. Asterisks indicate statistical significance after *post hoc* pairwise comparisons. (a) The average RNFL thickness is shown. (b) The temporal RNFL thickness is shown. (c) The RNFL thickness of temporal, superior, nasal, and inferior quadrants is shown for control eyes (black line) and for ipsilateral PD eyes (gray line). (d) RNFL thickness of temporal, superior, nasal, and inferior quadrants is shown for control eyes (black line) and for contralateral PD eyes (gray line).

Our study identified a pattern of axonal loss typically seen in LHON and DOA, where the temporal fibers belonging to the papillomacular bundle are characteristically susceptible [5]. Both diseases are associated with a complex I defect [5], which is also recognized as a key feature in the pathogenesis of PD, either in the sporadic cases or in the genetic forms [3,4], linking the current results with the pattern observed in LHON and DOA.

Optic nerve pathology in PD has been previously documented by others, some reporting a preferential loss of fibers in the infero-temporal quadrants, consistent with the involvement of the papillomacular bundle [9,10,13]. Macular and foveal abnormalities have also been documented [12]. However, Altintas

et al. [11] reported a significant RNFL thinning of the superior and nasal quadrants, whereas Archibald *et al.* [14] failed to find any differences between PD and controls.

The role of a specific PD neuropathology in the retina is controversial and clear evidence of alpha-synuclein deposition is still missing. The most consistent link we hypothesize between the subclinical optic nerve involvement in PD and the major mitochondrial optic neuropathies LHON and DOA is based on complex I dysfunction and altered mitochondrial dynamics [5]. This latter link is highlighted by mitochondrial network fragmentation in patient-derived cells from both DOA- and PD-affected individuals carrying, respectively, OPA1 and PINK1/Parkin mutations [3–5]. The balance between mitochondrial fission and fusion seems crucial for the correct execution of the quality control check, and excessive mitophagy has been documented in RGCs of OPA1 mutant mouse leading to their degeneration, as well as it is now a central theme in PD pathogenesis [4,17,18].

In conclusion, the current study implicates optic nerve in the neurodegenerative process of PD. The significant reduction in the temporal fibers suggests a mitochondrial pattern of retinal axonal loss in PD, supporting and in agreement with mitochondrial dysfunction in the pathogenesis of PD.

Disclosure of Conflict of Interest

Dr. La Morgia C has received financial support for meeting expenses by Zeiss. Dr. Barboni P reports no disclosure. Dr. Rizzo has received a research grant by the Fondazione Neureca Onlus. Dr. Carbonelli M reports no disclosure. Dr. Savini G reports no disclosure. Dr. Scaglione C has received honoraria for speaking engagements with Lundbeck and Boheringer and financial support for meetings by Ucb and Boheringer. Dr. Capellari S has received financial support for meeting expenses by Novartis. Dr. Bonazza S reports no disclosure. Dr. Giannoccaro MP reports no disclosure. Dr. Calandra-Buonaura G reports no disclosure. Prof Liguori R reports no disclosure. Prof Cortelli P has received honoraria for consulting activities with Allergan Italia, Boehringer Ingelheim Italia, Chelsea Therapeutics, GlaxoSmithKline S.p.A, Lundbeck Italy, Merck Sharp & Dohme (Italia), Teva, UCB Pharma S.p.A. Prof Martinelli P reports no disclosure. Prof Baruzzi A, reports no disclosure. Dr. Carelli V is consultant for Edison Pharmaceuticals and received research grants from the Italian Ministry of Health (RF-2009-1492481) and Telethon-Italy (#GGP11182).

References

1. de Lau LM, Breteler MM. Epidemiology of Parkinson's disease. *Lancet Neurol* 2006; **5:** 525–535.
2. Simuni T, Sethi K. Nonmotor manifestations of Parkinson's disease. *Ann Neurol* 2008; **64:** S65–S80.
3. Schapira AH. Mitochondria in the aetiology and pathogenesis of Parkinson's disease. *Lancet Neurol* 2008; **7:** 97–109.
4. Whitworth AJ, Pallanck LJ. The PINK1/Parkin pathway: a mitochondrial quality control system? *J Bioenerg Biomembr* 2009; **41:** 499–503.
5. Carelli V, La Morgia C, Valentino ML, Barboni P, Ross-Cisneros FN, Sadun AA. Retinal ganglion cell neurodegeneration in mitochondrial inherited disorders. *Biochim Biophys Acta* 2009; **1787:** 518–528.
6. Barboni P, Savini G, Valentino ML, *et al.* Retinal nerve fiber layer evaluation by optical coherence tomography in Leber's hereditary optic neuropathy. *Ophthalmology* 2005; **112:** 120–126.
7. Barboni P, Carbonelli M, Savini G, *et al.* Natural history of Leber's hereditary optic neuropathy: longitudinal analysis of the retinal nerve fiber layer by optical coherence tomography. *Ophthalmology* 2010; **117:** 623–627.
8. Bodis-Wollner I. Retinopathy in Parkinson Disease. *J Neural Transm* 2009; **116:** 1493–1501.
9. Inzelberg R, Ramirez JA, Nisipeanu P, Ophir A. Retinal nerve fiber layer thinning in Parkinson disease. *Vision Res* 2004; **44:** 2793–2797.
10. Yavas GF, Yilmaz O, Küsbeci T, Oztürk F. The effect of levodopa and dopamine agonists on optic nerve head in Parkinson disease. *Eur J Ophthalmol* 2007; **17:** 812–816.
11. Altintaş O, Işeri P, Ozkan B, Cağlar Y. Correlation between retinal morphological and functional findings and clinical severity in Parkinson's disease. *Doc Ophthalmol* 2008; **116:** 137–146.
12. Hajee ME, March WF, Lazzaro DR, *et al.* Inner retinal layer thinning in Parkinson disease. *Arch Ophthalmol* 2009; **127:** 737–741.
13. Moschos MM, Tagaris G, Markopoulos I, *et al.* Morphologic changes and functional retinal impairment in patients with Parkinson disease without visual loss. *Eur J Ophthalmol* 2011; **21:** 24–29.
14. Archibald NK, Clarke MP, Mosimann UP, Burn DJ. Retinal thickness in Parkinson's disease. *Parkinsonism Relat Disord* 2011; **17:** 431–436.
15. Gelb DJ, Oliver E, Gilman S. Diagnostic criteria for Parkinson disease. *Arch Neurol* 1999; **56:** 33–39.
16. Zucco G, Zeni MT, Perrone A, Piccolo I. Olfactory sensitivity in early-stage Parkinson patients affected by more marked unilateral disorder. *Percept Mot Skills* 2001; **92:** 894–898.
17. White KE, Davies VJ, Hogan VE, *et al.* OPA1 deficiency associated with increased autophagy in retinal ganglion cells in a murine model of dominant optic atrophy. *Invest Ophthalmol Vis Sci* 2009; **50:** 2567–2571.
18. Vives-Bauza C, Przedborski S. Mitophagy: the latest problem for Parkinson's disease. *Trends Mol Med* 2011; **17:** 158–165.