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## Distribution of Melatonin MT1 Receptor Immunoreactivity in Human Retina

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**SUMMARY** Melatonin is synthesized in the pineal gland and retina during the night. Retinal melatonin is believed to be involved in local cellular modulation and in regulation of light-induced entrainment of circadian rhythms. The present study provides the first immunohistochemical evidence for the localization of melatonin 1a-receptor (MT1) in human retina of aged subjects. Ganglion, amacrine, and photoreceptor cells expressed MT1. In addition, MT1 immunoreactivity was localized to cell processes in the inner plexiform layer and to central vessels of the retina, as well as to retinal vessels but not to ciliary or choroidal vessels. These results support a variety of cellular and vascular effects of melatonin in the human retina. Preliminary evidence from patients with Alzheimer's disease (AD) revealed increased MT1 immunoreactivity in ganglion and amacrine cells, as well as in vessels. In AD cases photoreceptor cells were degenerated and showed low MT1 expression.

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**KEY WORDS**

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ganglion cell  
photoreceptor  
amacrine cell  
vessels  
Alzheimer's disease

THE HORMONE MELATONIN, secreted from the pineal gland at night and suppressed by light during the day, provides a circadian and seasonal signal to the organism. The circadian rhythm is generated by the suprachiasmatic nucleus (SCN). Light information to the SCN is transduced via the retina, itself containing a circadian pacemaker (Terman et al. 1991; Herzog and Block 1999; Tosini 2000) which drives the rhythm of local melatonin synthesis. The function of melatonin in the human retina is not well known. In vertebrates, melatonin mediates dark-adaptive regulation of retinomotor movements (Pierce and Besharse 1985), circadian disc shedding in photoreceptor cells (White and Fisher 1989), regulation of horizontal cell sensitivity to light (Wiechmann et al. 1988), and modulation of dopamine release (Dubocovich 1983). The melatonin-synthesizing enzymes are found in photoreceptor cells, rods and cones (Wiechmann and Hollyfield 1989; Tosini 2000). As in the pineal, melatonin in the retina

may act as a signal of darkness. Melatonin has another characteristic. It is a highly potent antioxidative substance, reducing the damage caused by free radicals generated not only by light, but also by amyloid  $\beta$ -peptide, which is crucially involved in the neuropathology seen in several cognitively disabling neurodegenerative diseases such as Alzheimer's disease (AD) (Pappolla et al. 2000).

In mammals, the specific actions of melatonin are mediated by two different subtypes of G-protein-coupled receptors, the melatonin 1a (MT1) and melatonin 1b (MT2) receptors (Brydon et al. 1999; Dubocovich et al. 2000). Specific polyclonal antibodies are available against MT1 (Brydon et al. 1999). Its expression has been studied in rodents using immunocytochemistry and in situ hybridization (Fujieda et al. 1999, 2000). However, corresponding studies in humans remain to be done. In recent studies we were able to show localization of MT1 in humans in cerebrovascular tissue emphasizing melatonin's vasoactive properties (Savaskan et al. 2001) and in hippocampal neurons (Savaskan et al. in press).

The aim of this study was to provide immunohistochemical evidence, the first we are aware of, for the distribution of MT1 in human retina and in the ocular

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vascular system. In addition, eyes of two AD patients were included in the study as a preliminary investigation of possible alterations in the disease process.

## Materials and Methods

### Human Tissue

Paraffin-embedded human eyes fixed in 4% paraformaldehyde were obtained from seven subjects without neurological or psychiatric disease (six women and one man, mean age  $83.1 \pm 9.8$  years and two male AD cases, 83 and 84 years old. The mean postmortem delay was 21 hr and 31 min  $\pm$  9 hr and 10 min) for controls and 16 hr and 15 min for AD cases (Table 1). The diagnosis of AD was made with clinical evaluation and confirmed by postmortem neuropathological examination. The cause of death in most cases was heart failure or pneumonia, and pancreatitis for one control case (Table 1). The sample collection was approved by the Ethics Committee criteria and according to the Helsinki Declaration of 1975. Tissue samples were cut in the sagittal plane and 4- $\mu$ m-thick consecutive sections were made with a microtome.

### Immunohistochemistry

The sections were mounted on coated glass slides and deparaffinized. Endogenous peroxidase activity was blocked by bathing the sections in 80% methanol, 0.6% H<sub>2</sub>O<sub>2</sub> for 20 min at room temperature (RT). After several washes in PBS and preincubation in PBS with 3% blocking serum for 30 min the sections were reacted with primary antibody overnight at RT. The affinity-purified polyclonal antibody used to specifically detect MT1 was developed against a peptide (peptide 536) corresponding to a sequence found in the C-terminus of the receptor and the antibody recognition of native MT1 has been ascertained (Brydon et al. 1999). The non-commercial antibody was kindly provided by L. Brydon and R. Jockers (Institut Cochin de Génétique Moléculaire; Paris, France). In addition, the antibody has been applied successfully in previous immunohistochemical experiments (Savaskan et al. 2001; Savaskan et al. in press). The optimal concentration of the primary antibody was experimentally determined to be 1:100. In a second step, after several washes with PBS the sections were incubated with biotinylated secondary antibody (Vector Laboratories; Burlingame,

CA). The optimal dilution of the secondary antibody was 1:100. After several washes, a pre-formed ABC reagent (Vectastain; Vector Laboratories) was conjugated to the free biotin of the secondary antibody. The antigen was visualized using the peroxidase substrate 3-amino-9-ethylcarbazole (ACE). The samples were then counterstained with Mayer's hemalum. The sections were coverslipped with Kaiser's glycerol gelatin. To test the specificity of the antibody, control sections were stained simultaneously following the same procedure, with the exception that the primary antibody was omitted. All sections were assessed for localization and intensity of specific immunoreactivity on a semiquantitative scale of +/+++ by two blinded observers (Table 2).

## Results

The cytoarchitectural classification regarding cells, different layers, and vascularization in this section follows detailed descriptions by Spencer (1996).

### Cellular Structures

Different cells within the retina displayed specific MT1 immunoreactivity (Figures 1A–1D), whereas adjacent control sections omitting the primary antibody revealed no immune reaction (Figures 1E and 1F). The majority of ganglion cells were immunoreactive for MT1 in their somata and dendritic processes, both in controls and in AD cases (Figures 1A and 1C). Staining was homogeneously distributed in the perinuclear area. In five control cases (Table 1), single MT1-immunoreactive amacrine cells were located in the inner nuclear layer immediately adjacent to the inner plexiform layer. Only a minor subset of amacrine cells were stained. In all cases there was a slight granular MT1 labeling in the inner plexiform layer located on cell processes. The outer plexiform layer and the horizontal cells were not immunolabeled. Photoreceptor cells revealed distinct MT1 immunoreactivity in their inner segments (Figure 1B). Cell somata and outer seg-

**Table 2** Distribution and intensity of MT1 immunoreactivity in human retina in controls (C) and Alzheimer's disease patients (AD)<sup>a,b</sup>

		Cell structures			Vessels			
		PC	GC	AC	CA	CV	RV	CI
C	1	+++	++	+	+	+	+	-
	2	+++	++	+	++	++	+	-
	3	++	+	-	++	++	++	-
	4	++	++	+	+	-	+	-
	5	+	+	+	++	++	+	-
	6	++	+	+	++	++	+	-
	7	++	+	-	++	++	+	-
AD	1	+	+++	+++	+++	+++	++	-
	2	+	+++	++	+++	+++	++	-

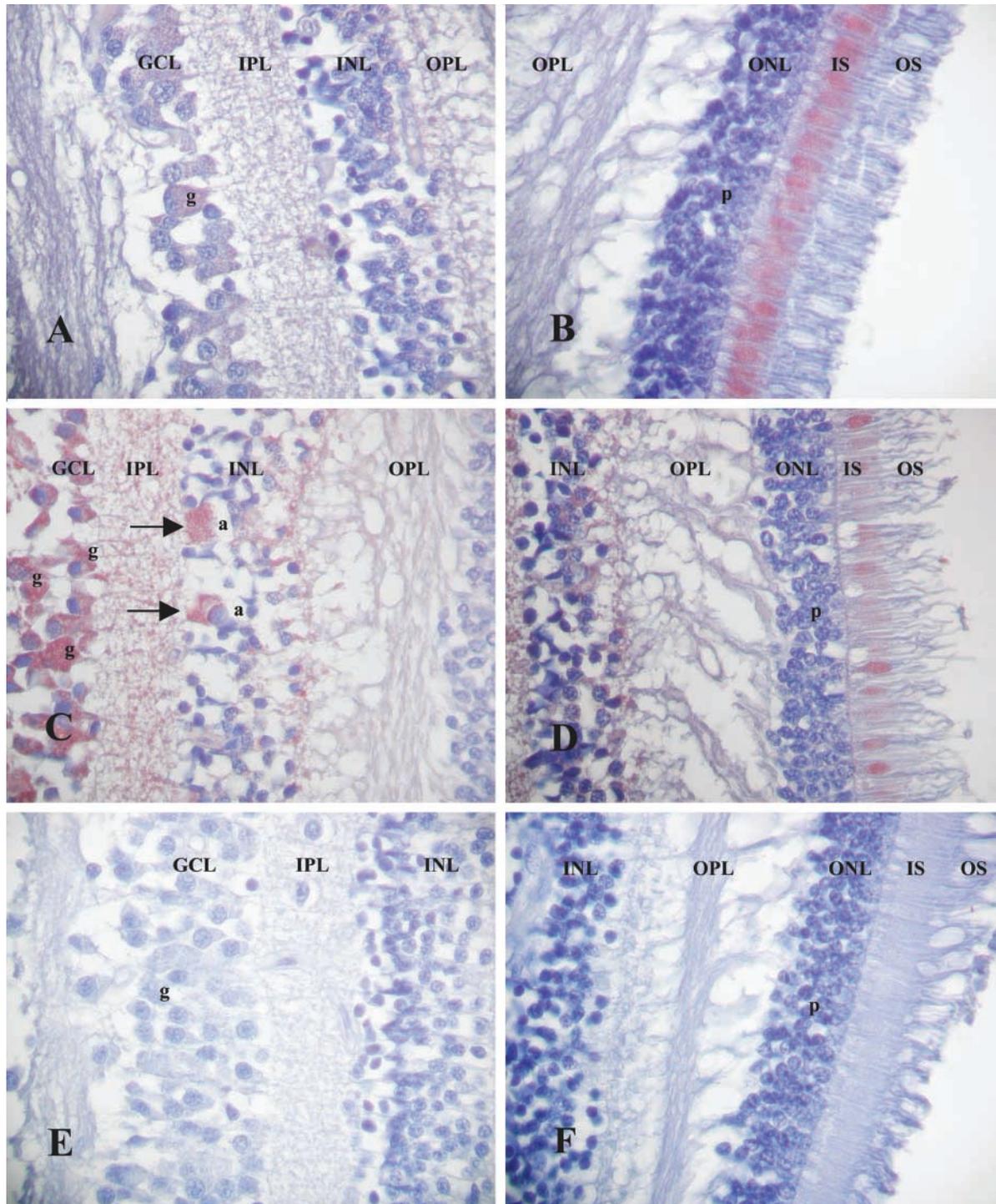
<sup>a</sup>MT1 staining intensity: -, no immunoreactivity; +, slight; ++, moderate; +++, high.

<sup>b</sup>PC, photoreceptor cells; GC, ganglion cells; AC, amacrine cells; CA, central artery; CV, central vein; RV, retinal vessels; CI, ciliary vessels.

**Table 1** Data of control (C) and Alzheimer's disease (AD) cases<sup>a</sup>

Case no.	Clinical diagnosis	Age	Gender	PD	CD
1	C	69	F	20 hr	Pancreatitis
2	C	76	F	9 hr 40 min	Heart failure
3	C	88	F	31 hr	Heart failure
4	C	76	F	9 hr	Pneumonia
5	C	85	M	31 hr	Heart failure
6	C	97	F	26 hr	Heart failure
7	C	91	F	24 hr	Heart failure
1	AD	83	M	15 hr	Heart failure
2	AD	84	M	17 hr 30 min	Pneumonia

<sup>a</sup>PD, postmortem delay; CD, cause of death.



**Figure 1** Sagittal sections through the human retina. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nucleiform layer; OPL, outer plexiform layer; ONL, outer nucleiform layer consisting of cell somata of photoreceptor cells; IS, inner segments; OS, outer segments of photoreceptor cells; g, ganglion cells; a, amacrine cells; p, photoreceptor cells. (A) Ganglion cells show MT1 immunoreactivity as a red deposit in a control case. (B) The IS of photoreceptor cells are distinctly immunopositive for MT1 in a control case. (C) The staining intensity is increased in an AD case. Note that the IPL, and not the OPL, exhibits MT1 immunoreactivity, and some amacrine cells are strongly MT1 positive, as indicated by arrows. (D) The MT1 staining intensity in photoreceptor cells is decreased in an AD case. (E,F) Control sections stained simultaneously following the same procedure, with the exception that the primary antibody was omitted. The lack of MT1 immunoreactivity confirms the specificity of the reaction. Original magnification  $\times 242$ .

ments were excluded. All photoreceptor cells were stained so that MT1 extended as an immunoreactive band throughout the photoreceptor cell layer.

The two AD cases showed the following differences in the distribution of MT1 immunoreactivity. First, the staining intensity and the numbers of MT1-immunoreactive cells were distinctly increased in ganglion cells and inner plexiform cell layers (Figure 1C). Second, both AD cases revealed well-stained amacrine cells, and the number of these MT1-immunoreactive cells was twice that in controls (Figure 1C). There was also increased staining intensity within the inner plexiform layer. A decreased MT1 staining in the highly degenerated photoreceptor cell layer could be observed (Figure 1D). Therefore, in AD cases only single cells were immunoreactive for MT1 so that there was no continuous band of immunoreactivity throughout the entire photoreceptor cell layer as in controls (Figures 1B and D).

#### Ocular Vessels

In all control eyes a distinctly positive MT1 immunoreactivity in the adventitia of central retinal arteries and veins could be observed (Figures 2A and 2B). The same staining pattern was found even in the small retinal vessels located in the ganglion cell layer and inner plexiform layer (Figure 2E). Both ciliary and choroidal vessels were devoid of MT1 immunoreactivity (Figure 2F). In AD, increased MT1 staining was detected in central arteries and veins and in retinal vessels, whereas ciliary and choroidal vessels were not stained (Figures 2D and 2E).

#### Discussion

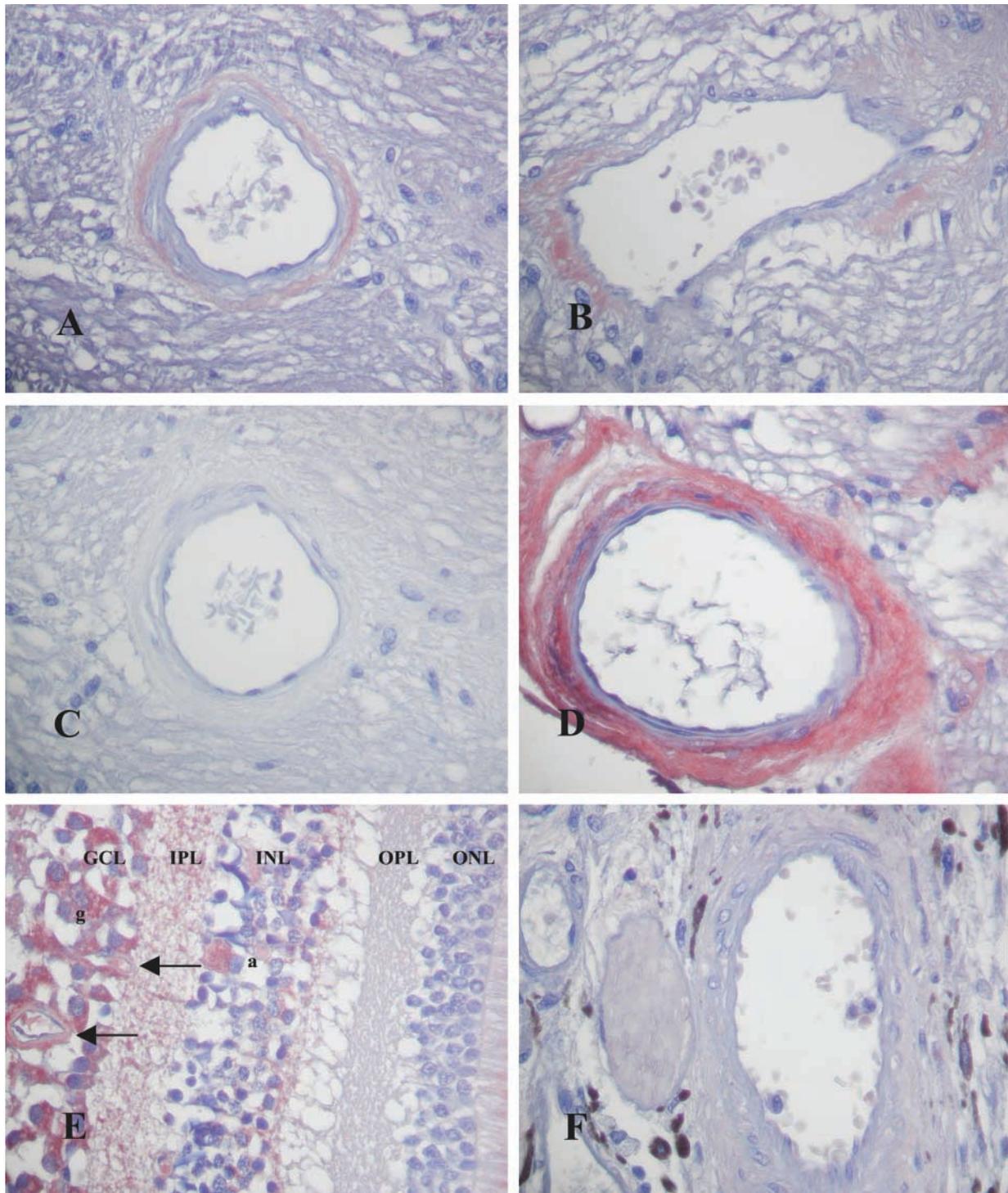
The present study has demonstrated the localization of MT1 in cellular structures of the retina and in ocular vessels of aged humans, and has provided preliminary evidence for alterations in retinal MT1 expression in AD patients. After previous demonstration of melatonin, its synthesizing enzymes (Bubenik et al. 1974; Vivien-Roels et al. 1981; Wiechmann and Hollyfield 1989; Tosini 2000), and MT1 receptors in vertebrate retina (Fujieda et al. 1999,2000), this is the first demonstration of MT1 distribution in human retina.

Several types of retinal cells displayed MT1 immunoreactivity. The localization of MT1 to ganglion and amacrine cells is in accordance with previous reports in rodent retina (Fujieda et al. 1999,2000), where these cells are believed to be major sites of melatonin action. Because MT1-positive amacrine cells have been found to be dopaminergic and GABAergic by double-immunolabeling experiments, a direct regulatory action of melatonin on dopamine release by enhancing GABA activity has been postulated (Fujieda et al. 2000). Also in agreement with rodent studies, the

strongly positive MT1 staining in the inner plexiform layer has been suggested to correspond to melatonin binding sites on dendritic processes of amacrine and ganglion cells (Fujieda et al. 1999,2000). In our series, no MT1 expression was detected in the outer plexiform layer or in the horizontal cells, similar to that in ganglion and amacrine cells. This contrasts to the strongly MT1-positive staining in the outer plexiform layer and horizontal cells in rat retina (Fujieda et al. 1999), which may reflect either interspecies differences or tissue-specific features.

Another contrast to rodents (Fujieda et al. 1999,2000) was the distinct MT1 staining in the inner segments of photoreceptor cells in elderly humans. Melatonin activates disc shedding in rodent photoreceptor cells (White and Fisher 1989). Melatonin has been located in the inner segments of photoreceptor cells and reaches a peak level in outer segments at the end of the night, which may be related to melatonin's protective action counteracting light's toxic effects (Hsu and Molday 1994; Marchiafava and Longoni 1999). Therefore, melatonin may be essential for photoreceptor cells. Our results indicate that, at least in aged humans, external melatonin sources may also play an important role in the maintenance of photoreceptor cells.

Although only two AD cases were investigated, the MT1 alterations were notable. The increase of MT1 immunoreactivity in ganglion and amacrine cells may indicate receptor upregulation, possibly as a regulative response to declining nocturnal melatonin levels in elderly subjects and in AD (Ferrari et al. 2000), as we have already found in hippocampal neurons of AD patients (Savaskan et al. in press). Previous data localizing MT1 to both dendrites and axons of ganglion cells raised the possibility that retinal melatonin may act on dendritic processes, whereas pineal melatonin may stimulate ganglion cell terminals in their target areas (Fujieda et al. 2000). Therefore, pineal melatonin's feedback to the SCN has been assumed to occur both directly and indirectly via ganglion cells. Ganglion cells may be influenced by both pineal and retinal melatonin (Fujieda et al. 2000). This emphasizes a role for melatonin in ganglion cells in modifying retinal sensitivity and thus contributing to entrainment of rhythms driven by the SCN (Fujieda et al. 2000). Mice completely lacking photoreceptor cells showed unattenuated phase-shifting responses to light, suggesting that novel ocular photoreceptors, possibly in ganglion and amacrine cells, may be active in circadian regulation (Freedman et al. 1999; Lucas et al. 1999). MT1 upregulation in retinal cells in AD may be related to altered photic input to the SCN, which itself is diminished in function in AD (Swaab et al. 1985), thus additionally contributing to the enhanced incidence of circadian rhythm disturbances in these subjects.



**Figure 2** The vessels of the retina. (A) The central artery and (B) vein of controls display MT1 immunoreactivity as a red deposit. (C) The control section (adjacent to A) omitting the primary antibody reveals no MT1 immunoreactivity. (D) The intensity of the vascular MT1 staining is distinctly increased in AD. (E) Retinal arteries are also immunopositive for MT1 as indicated by arrow (an AD case). (F) The choroidal vessels show no MT1 immunoreactivity (an AD case). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nucleiform layer; OPL, outer plexiform layer; ONL, outer nucleiform layer; g, ganglion cells; a, amacrine cells. Original magnification  $\times 242$ .

Free radicals are generated in mitochondria owing to their high respiratory activity (Demontis et al. 1997) and by light absorption (Remé et al. 1996). Melatonin, as a highly effective antioxidant scavenging free radicals, may therefore be essential for photoreceptor cells that are prone to generation of reactive oxygen species (Marchiafava and Longoni 1999). On the other hand, there is contrary evidence about the protective role of melatonin within the retina. Systemic administration of melatonin increases retinal light damage and the melatonin receptor competitive antagonist luzindole protects photoreceptor cells from light damage (Sugawara et al. 1998). Melatonin's modulatory action on dopaminergic transmission has been postulated to regulate susceptibility to light damage (Iuvone and Gan 1995; Sugawara et al. 1998). Therefore, the decrease in MT1 immunoreactivity in photoreceptor cells in AD may be related to the marked degenerative changes found in these cells.

In addition to cellular structures, central and retinal vessels were also immunoreactive for MT1 in their adventitia. Melatonin is a potent vasoactive substance regulating cerebrovascular responsiveness and blood flow in brain (Régriny et al. 1998). Melatonin administration induces cerebral vasoconstriction, resulting in improved dilatory capacity (Régriny et al. 1998). The vascularization of the retina arises from two different sources, both originating from the arteria ophthalmica. Whereas the arteria centralis retinae supplies the inner layers, including ganglion cell and inner plexiform layers, ciliary and choroidal vessels are responsible for blood circulation in outer layers (Spencer 1996). The localization of MT1 was distinctly restricted to the vascular network of the retinal vessel system. MT1 is localized to the vascular adventitia and not to the smooth muscle layer (Savaskan et al. 2001). The adventitial localization of MT1 suggests an indirect action of melatonin on smooth muscle of the vasculature. The upregulation of vascular MT1 expression in AD retina parallels our recent findings in cerebrovascular MT1 immunoreactivity (Savaskan et al. 2001). Cerebral hypoperfusion plays a decisive role in initiating neurodegeneration in AD (De la Torre and Stefano 2000). Therefore, the observed alterations in vascular MT1 expression in other brain regions in AD (Savaskan et al. 2001) may be accompanied by corresponding changes in retina.

Taken together, the results provide evidence that melatonin may be involved in different cellular and vascular processes in the human retina via MT1. The evaluation of a larger number of cases will be necessary to confirm the striking alterations in AD. Understanding the effects of melatonin in retina as well as brain may be important to combat neurodegeneration and circadian disturbances in AD.

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## Literature Cited

- Brydon L, Roka F, Petit L, De Coppet P, Tissot M, Barrett P, Morgan PJ, Nanoff C, Strosberg AD, Jockers R (1999) Dual signaling of human Mel1a melatonin receptors via G<sub>12</sub>, G<sub>13</sub> and G<sub>q/11</sub> proteins. *Mol Endocrinol* 13:2025–2038
- Bubenik GA, Brown GM, Uhlir I, Grota LJ (1974) Immunohistological localization of N-acetyldolalkylamines in pineal gland, retina and cerebellum. *Brain Res* 81:233–242
- De la Torre JC, Stefano GB (2000) Evidence that Alzheimer's disease is a microvascular disorder: the role of constitutive nitric oxide. *Brain Res Rev* 34:119–136
- Demontis GC, Longoni B, Gargini C, Cervetto L (1997) The energetic cost of photoreception in retinal rods of mammals. *Arch Ital Biol* 135:95–109
- Dubocovich ML (1983) Melatonin is a potent modulator of dopamine release in the retina. *Nature* 306:782–784
- Dubocovich ML, Masana MI, Benloucif S (2000) Molecular pharmacology and function of melatonin receptor subtypes. In Olcese J, ed. *Melatonin after Four Decades*. New York, Kluwer Academic/Plenum Publishers, 181–190
- Ferrari E, Arcaini A, Gornati R, Pelanconi L, Cravello L, Fioravanti M, Solerte SB, Magri F (2000) Pineal and pituitary-adrenocortical function in physiological aging and in senile dementia. *Exp Gerontol* 35:1239–1250
- Freedman MS, Lucas RJ, Soni B, Von Schantz M, Muñoz M, David-Gray Z, Foster R (1999) Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors. *Science* 284:502–504
- Fujieda H, Hamadanizadeh SA, Wankiewicz E, Pang SF, Brown GM (1999) Expression of mt<sub>1</sub> melatonin receptor in rat retina: evidence for multiple cell targets for melatonin. *Neuroscience* 93:793–799
- Fujieda H, Scher J, Hamadanizadeh SA, Wankiewicz E, Pang SF, Brown GM (2000) Dopaminergic and GABAergic amacrine cells are direct targets of melatonin: immunocytochemical study of mt<sub>1</sub> melatonin receptor in guinea pig retina. *Vis Neurosci* 17: 63–70
- Herzog ED, Block GD (1999) Keeping an eye on retinal clocks. *Chronobiol Int* 16:229–247
- Hsu S-C, Molday RS (1994) Glucose metabolism in photoreceptor outer segments. *J Biol Chem* 269:17954–17959
- Iuvone PM, Gan J (1995) Functional interaction of melatonin receptors and D1 dopamine receptors in cultured chick retinal neurons. *J Neurosci* 15:2179–2185
- Lucas RJ, Freedman MS, Muñoz M, García-Fernández J-M, Foster RG (1999) Regulation of the mammalian pineal by non-rod, non-cone, ocular receptors. *Science* 284:505–507
- Marchiafava PL, Longoni B (1999) Melatonin as an antioxidant in retinal photoreceptors. *J Pineal Res* 26:184–189
- Pappolla MA, Chyan Y-J, Poeggeler B, Frangione B, Wilson G, Ghiso J, Reiter RJ (2000) An assessment of the antioxidant and the antiamyloidogenic properties of melatonin: implications for Alzheimer's disease. *J Neural Transm* 107:203–231
- Pierce ME, Besharse JC (1985) Circadian regulation of retinomotor movements. I. Interaction of melatonin and dopamine in the control of cone length. *J Gen Physiol* 86:671–689
- Régriny O, Delagrangé P, Scalbert E, Atkinson J, Lartaud-Idjouadienne I (1998) Melatonin improves cerebral circulation security margins in rats. *Am J Physiol* 275:H139–144
- Remé CE, Hafezi F, Reinboth J, Clausen M (1996) Light damage revisited: converging evidence, diverging views? *Graefes Arch Clin Exp Ophthalmol* 234:2–11

- Savaskan E, Olivieri G, Brydon L, Jockers R, Kräuchi K, Wirz-Justice A, Müller-Spahn F (2001) Cerebrovascular melatonin MT1-receptor alterations in patients with Alzheimer's disease. *Neurosci Lett* 308:9–12
- Savaskan E, Olivieri G, Meier F, Brydon L, Jockers R, Ravid R, Wirz-Justice A, Müller-Spahn F (in press) Increased melatonin 1a-receptor immunoreactivity in the hippocampus of Alzheimer's disease patients. *J Pineal Res*
- Spencer WH (1996) *Ophthalmic Pathology*. 4th ed. Philadelphia, WB Saunders
- Sugawara T, Sieving PA, Iuvone PM, Bush RA (1998) The melatonin antagonist luzindole protects retinal photoreceptors from light damage in rat. *Invest Ophthalmol Vis Sci* 39:2458–2465
- Swaab DF, Fliers E, Partiman TS (1985) The suprachiasmatic nucleus of the human brain in relation to sex, age and senile dementia. *Brain Res* 342:37–44
- Terman M, Remé CE, Wirz-Justice A (1991) The visual input stage of the mammalian circadian pacemaking system: II. The effect of light and drugs on retinal function. *J Biol Rhythms* 6:31–48
- Tosini G (2000) Melatonin circadian rhythm in the retina of mammals. *Chronobiol Int* 17:599–612
- Vivien-Roels B, Pévet P, Dubois MP, Arendt J, Brown GM (1981) Immunohistochemical evidence for the presence of melatonin in the pineal gland, the retina and the Harderian gland. *Cell Tissue Res* 217:105–115
- White MP, Fisher LJ (1989) Effects of exogenous melatonin on circadian disc shedding in the albino rat retina. *Vis Res* 29:167–179
- Wiechmann AF, Hollyfield JG (1989) HIOMT-like immunoreactivity in the vertebrate retina: a species comparison. *Exp Eye Res* 49:1079–1095
- Wiechmann AF, Yang XL, Wu SM, Hollyfield JG (1988) Melatonin enhances horizontal cell sensitivity in salamander retina. *Brain Res* 453:377–380

