Published in final edited form as:

Exp Eye Res. 2017 December ; 165: 90–95. doi:10.1016/j.exer.2017.09.007.

Melatonin signaling affects the timing in the daily rhythm of phagocytic activity by the retinal pigment epithelium

Virgine Laurent^a, Anamika Sengupta^b, Aída Sánchez-Bretaña^b, David Hicks^a, and Gianluca Tosini^{b,*}

^aInstitut des Neurosciences Cellulaires et Integratives (INCI), CNRS UPR3212, 5 rue Blaise Pascal, 67084 Strasbourg, France

^bNeuroscience Institute, Department of Pharmacology and Toxicology, Morehouse School of Medicine, Atlanta, USA

Abstract

Earlier studies in *Xenopus* have indicated a role for melatonin in the regulation of retinal disk shedding, but the role of melatonin in the regulation of daily rhythm in mammalian disk shedding and phagocytosis is still unclear. We recently produced a series of transgenic mice lacking melatonin receptor type 1 (MT₁) or type 2 (MT₂) in a melatonin-proficient background and have shown that removal of MT₁ and MT₂ receptors induces significant effects on daily and circadian regulation of the electroretinogram as well as on the viability of photoreceptor cells during aging. In this study we investigated the daily rhythm of phagocytic activity by the retinal pigment epithelium in MT₁ and MT₂ knock-out mice. Our data indicate that in MT₁ and MT₂ knock-out mice the peak of phagocytosis is advanced by 3 h with respect to wild-type mice and occurred in dark rather than after the onset of light, albeit the mean phagocytic activity over the 24-h period did not change among the three genotypes. Nevertheless, this small change in the profile of daily phagocytic rhythms may produce a significant effect on retinal health since MT₁ and MT₂ knock-out mice showed a significant increase in lipofuscin accumulation in the retinal pigment epithelium.

Keywords

Melatonin; RPE; Circadian; Photoreceptors; Phagocytosis

Retinal pigment epithelium (RPE) is involved in many physiological functions that are necessary to maintain normal photoreceptor function (Bok, 1993). One of the most important and fascinating roles played by the RPE is phagocytosis of the membrane disks that are shed by photoreceptor outer segments (Nguyen-Legros and Hicks, 2000). Although disk shedding and phagocytosis occur throughout the day, a significant increase in phagocytic activity by the RPE cells is observed every day at 1–2 h after the onset of light (La Vail, 1976; Grace et al., 1999). These rhythms persist in constant darkness conditions, thus demonstrating that they are under the control of a circadian clock (La Vail, 1976;

*Corresponding author. gtosini@msm.edu (G. Tosini).

Teirstein et al., 1980; Terman et al., 1993; Grace et al., 1999). Additional studies indicate that the circadian clock controlling disk shedding is located within the eye (La Vail, 1976; Teirstein et al., 1980), but it is not known whether this rhythm is controlled by the circadian clock present in the retina (Tosini and Menaker, 1996, 1998; Ruan et al., 2008; Tosini et al., 2007), and/or in the RPE (Baba et al., 2010, 2017; Ruggiero et al., 2012).

In the retina, clock-controlled melatonin production is principally generated in the photoreceptors at night (Tosini and Menaker, 1996, 1998; Liu et al., 2004). In the mouse retina melatonin modulates the daily and circadian rhythm in visual processing (Baba et al., 2009, 2013; Sengupta et al., 2011), photoreceptor viability during aging (Gianesini et al., 2016), and delays photoreceptor degeneration in two mouse models of retinitis pigmentosa (Liang et al., 2001; Xu et al., 2017). A few clinical studies have also reported that melatonin production is decreased in patients affected by age-related macular degeneration (Rosen et al., 2009), and daily administration of melatonin may delay the progression of this disease (Yi et al., 2005). Hence melatonin is an important player in retinal physiology and pathophysiology (Tosini et al., 2012).

Previous investigations have demonstrated that melatonin binds to two different types of G-protein-coupled receptors named MT₁ and MT₂ (Tosini et al., 2014). Both of these receptors have been identified in the mouse retina. MT₁ receptors are present in photoreceptors, inner retinal neurons and retinal ganglion cells (Baba et al., 2009; Sengupta et al., 2011), whereas MT₂ receptors are present in photoreceptors and inner retinal neurons, but not in retinal ganglion cells (Baba et al., 2013). In rat, MT₁ receptors have been detected in the RPE (Fujieda et al., 1999), and in *Xenopus* the mRNAs encoding for both melatonin receptors have been found in the RPE (Wiechmann and Summers, 2008). Interestingly, it has been reported that in rat RPE, administration of exogenous melatonin induced up-regulation of 15 genes and down-regulation of 2 genes (Wiechmann, 2002). Moreover administration of melatonin induced disk shedding in *Xenopus* retina (Besharse and Dunis, 1983) and – in rat – provoked an increase in the number of large phagosomes in RPE cells (White and Fisher, 1989). However, it is worth mentioning that rhythmic disk shedding is also present in mice that are incapable of synthesizing substantial quantities of melatonin (Grace et al., 1999).

Melatonin proficient-mice (C3H-*f*^{+/+}) and C3H-*f*^{+/+} in which MT₁ (C3H/*f*^{+/+}MT₁^{-/-}) and MT₂ (C3H/*f*^{+/+}MT₂^{-/-}) were genetically ablated were used in this study (see Baba et al., 2013 for details). Mice were maintained in a 12-h Light: Dark (LD) cycle (lights on at 6 am (denoted as Zeitgeber Time (ZT) 0) and lights off at 6pm (ZT12), food and water were available ad libitum. All the experimental procedures were carried out in accordance with Association for Assessment of Laboratory Animal Care policies and approved by the Morehouse School of Medicine Animal Care and Use Committee.

The eyes of C3H-*f*^{+/+} or C3H-*f*^{+/+}-MT₁^{-/-} (MT₁) or C3H-*f*^{+/+}-MT₂^{-/-} MT₂^{-/-} mice were collected every 3 h over a 24-h period and fixed in paraformaldehyde 4%, and then stored in PBS at 4 °C. Prior to cryoprotection, eyes were dissected into two halves at the level of the optic nerve, removing the cornea, lens and aqueous humor. Both halves were then incubated successively in a 10% sucrose PBS solution followed by a 20% sucrose solution and finally

in a 30% sucrose bath, -hour each. Each semi-globe was individually included in Tissue-Tek® OCT™ compound and frozen in liquid nitrogen before being kept at -80°C . The cryoprotected eyes were cut into $10\text{ }\mu\text{m}$ slices using a cryostat (Leica CM3050 S) prior to performing immunohistochemistry for phagosome detection. Slides with retina and RPE slices (4 for each genotype at every time point) were thawed, dried and sections were permeabilized 5 min in a 0.1% Triton X-100 solution (Sigma). Slices were next immersed in blocking buffer (3% Bovine serum albumin, 0.1% Tween-20 (Euromedex), 0.1% sodium azide in PBS) for 1 h. Then, sections were incubated with blocking buffer and anti-rhodopsin antibody Rho4D2 (Hicks and Molday, 1985), at a 1/40000 dilution overnight at 4°C . The following day, sections were washed with PBS for 1 h before the application of the secondary antibody Alexa 488 1/400 and DAPI 1/400 diluted in blocking buffer. Sections were thus incubated for 2 h and washed in PBS for 45 min. Slides were mounted in Glycerol/PBS 1:1 prior to observation under a fluorescence microscope. DAPI staining allowed precise visualization of retinal-RPE morphology, while phagosome counting was rendered possible by rhodopsin immunodetection (Fig. 1A). For each mouse, 4 sections were analyzed. Each section was divided into 21 grids of $150\text{ }\mu\text{m}$ on which phagosomes (defined as immunofluorescent inclusions of $>1\text{ }\mu\text{m}$ – see (Bobu and Hicks, 2009 for details) were manually identified and counted. Data presented are the means of the no. of phagosomes per $150\text{ }\mu\text{m}$ retinal section. The observer was blind to the time of day at which sections were obtained and the genotype.

To determine the level of lipofuscin in the RPE, cryosections of eyecups from 3- and 18-month-old mice of the three genotypes ($n = 3$ for each genotype, 4 randomly selected sections per animal) were examined by fluorescence microscopy (Zeiss Axioskop) to detect DAPI-stained nuclei, and autofluorescence was detected in the rhodamine channel as described by Nandrot et al. (2004).

Quantification of phagosomes as a function of the time of the day showed that phagocytic activity was rhythmic in all three genotypes (One-Way ANOVA, $P < 0.01$ in all cases, Fig. 1B, D). In C3H- $f^{+/+}$ phagocytic activity peaked 1 h after light onset, as seen in previous studies in mice and other mammals (ZT1; Fig. 1B). Phagosome counts were lower for the other points, reaching a minimum in early night (ZT16; Fig. 1B). In both $MT_1^{-/-}$ and $MT_2^{-/-}$ mice the peak of phagocytic activity occurred at ZT 22 (i.e., 3 h before that in C3H- $f^{+/+}$) while the minimum was observed at ZT16 (Fig. 1C and, D). To determine rhythmicity in the daily phagocytic activity we performed a COSINOR analysis (see Hiragaki et al., 2014 for details). As shown in Table 1 a significant daily rhythm ($P < 0.05$) was detected in all the three genotypes. In $MT_1^{-/-}$ and $MT_2^{-/-}$ the acrophase of the peak was advanced with respect to what observed in C3H- $f^{+/+}$, whereas no difference were observed in the mesor and amplitude. However, it worth noting that the total number of phagosome for each measured time point over the entire 24-hour period demonstrated that total mean phagocytic activity did not change among the three genotypes (C3H- $f^{+/+} = 18.91 \pm 1.669$ (SEM); $MT_1^{-/-} = 18.47 \pm 1.42$; $MT_2^{-/-} = 18.17 \pm 1.42$; ANOVA, $P > 0.5$).

Fig. 2A–F shows representative photomicrographs of sections obtained from the eyes of mice of the three different genotypes and at the two different ages. Fig. 3A–F shows representative images obtained from the eyecups of young (3–4 months) C3H- $f^{+/+}$ (A), $MT_1^{-/-}$ (B) $MT_2^{-/-}$ (C) and aged (17–18 months) C3H- $f^{+/+}$ (D) and $MT_1^{-/-}$ and $MT_2^{-/-}$ (F) mice. Analysis of the images indicated that the RPE of $MT_1^{-/-}$ and $MT_2^{-/-}$ mice contained higher numbers of vesicular autofluorescent storage bodies with respect to what was observed in the RPE of C3H- $f^{+/+}$ mice of a similar age.

As previously mentioned the existence of daily and circadian rhythms in photoreceptor disk shedding and phagocytosis are well documented (La Vail, 1976; Teirstein et al., 1980; Terman et al., 1993; Grace et al., 1999), but until recently it was not clear whether maintenance of these rhythms was important for photoreceptor or RPE health. The importance of the daily burst in phagocytic activity for photoreceptors and RPE health was recently demonstrated by study in which it was reported that mice lacking the daily burst of phagocytosis ($\alpha\beta5$ integrin receptors knock-out mice) show age-related loss of photoreceptor function and lipofuscin accumulation in the RPE (Nandrot et al., 2004).

Our data expand these previous studies by showing that lack of MT_1 or MT_2 receptors affects the timing of the daily rhythm in phagocytic activity and may lead to lipofuscin accumulation in aged mice. Interestingly, the removal of either MT_1 or MT_2 receptors produced a similar effect on RPE phagocytic activity, thus suggesting that - as seen for electroretinogram - the action of melatonin on phagocytic activity is mediated by MT_1/MT_2 heteromers (Baba et al., 2013). As we observed a 3-h anticipated shift in both KO mice models, we do not measure a widespread disruption of the phagocytic peak. This aspect has been tested with a COSINOR analysis (Table 1) of the RPE phagocytic activity. Although melatonin receptor transcripts or immunoreactivity in the RPE have been reported for a few species (see Wiechmann and Summers, 2008 for recent review), in our previous studies we did not detect MT_1 or MT_2 mRNA (Baba et al., 2009, 2013) or MT_1 immunoreactivity (Sengupta et al., 2011) in mouse RPE. Moreover, we have assessed the clock markers in both $MT_1^{-/-}$ and $MT_2^{-/-}$ genotypes compared to C3H- $f^{+/+}$, but no change could be observed in the clock genes expression indicating that the RPE clock is not involved and the total number of phagosomes remains similar in the 3 genotypes over 24h (unpublished data). Hence our data suggest that melatonin does not directly act on the RPE, but rather its action may be mediated by photoreceptors where these receptors have been localized (Baba et al., 2013). In a previous publication, we have analyzed the expression of 9 different transcriptional regulators involved in the retinal physiology (Kunst et al., 2015) in $MT_1^{-/-}$, $MT_2^{-/-}$ and C3H- $f^{+/+}$ mice. One of them is a possible candidate for the phase-shifting/spreading of the phagocytosis peak: *Pgc-1* (energy metabolism) because its expression is no longer rhythmic in the $MT_1^{-/-}$. However, if *Pgc-1* was involved in the process described here, we should observe two different profiles of the phagocytic activity in $MT_1^{-/-}$ and $MT_2^{-/-}$ mouse RPE compared to WT mice, which we do not see. As mentioned earlier, $MT_2^{-/-}$ are not present on RPE cells, Hence MT_2 signaling cannot act on *Pgc-1* expression although our present data suggest the role of heterodimerized melatonin receptors. Another

non-explored possibility is the link that melatonin may represent between the circadian clock and the newly discussed metabolic/biochemical clock (Milev and Reddy, 2015). Melatonin is known to exert an antioxidant effect, as physiological and pharmacological concentrations have been observed to up-regulate antioxidant enzymes through membrane receptors (Tosini et al., 2014). Therefore, melatonin may use the high metabolic status of the cell (daily photoreceptor tip renewal, phototransduction) and potential targets (antioxidant enzymes) to participate in the phagocytosis event.

Although previous studies have shown that melatonin signaling may be implicated in the regulation of the daily rhythm in photoreceptor disk shedding and phagocytosis (Besharse and Dunis, 1983; White and Fisher, 1989), other studies have shown that melatonin is not required to generate daily RPE phagocytosis since this rhythm is present in melatonin-proficient (Grace et al., 1999) and in melatonin-deficient mice (Grace et al., 1999; Nandrot et al., 2004). Regarding the apparent conflict between most published articles on photoreceptor phagocytosis and the data described by Grace et al. (1999) in melatonin “deficient” mice, we may emit the following hypothesis: Melatonin is not absent in the retina of these animals but rather not rhythmically expressed due to a mutation in the expression of *Arylalkylamine N-acetyltransferase (Aanat*, the limiting enzyme of melatonin synthesis). As mentioned above, the clock does not seem to control the phagocytosis phase/synchronization in the RPE. Therefore, the presence of non-rhythmic melatonin signaling may be sufficient to elicit the cascade of events necessary for phagocytosis to happen in the RPE. Our new data expand these previous investigations by demonstrating that – in a melatonin-proficient mouse - melatonin signaling is necessary for correct timing of the daily burst in RPE phagocytic activity.

In conclusion, the results presented in this study suggest that just a 3 h advance in the peak of phagocytic activity correlates with increased lipofuscin accumulation in the RPE, thus producing a phenotype resembling that described in $\alpha\text{v}\beta 5$ integrin knock-out mice (Nandrot et al., 2004). Hence our new study indicates that timing of the daily burst in RPE phagocytic activity may be also important for photoreceptor health. Our study also suggests that precise coordination of the different steps involved in recognition, ingestion and degradation of outer segment materials may be essential for optimal clearance of debris, and possibly misalignment of these processes, as seems to occur in $\text{MT}_1^{-/-}$ and $\text{MT}_2^{-/-}$ mice, may lead to gradual increases in lipofuscin accumulation and decreased cell viability.

Acknowledgments

This work was supported by grants from the National Institutes of Health Grants EY022216, EY026291 to G.T. and by 5U54NS083932, S21MD000101, G12-RR03034, U54RR026137 to Morehouse School of Medicine.

References

- Baba K, Pozdeyev N, Mazzoni F, Contreras-Alcantara S, Liu C, Kasamatsu M, Martinez-Merlos T, Strettoi E, Iuvone PM, Tosini G, 2009 Melatonin modulates visual function and cell viability in the mouse retina via the MT1 melatonin receptor. *Proc. Natl. Acad. Sci. U. S. A* 106, 15043–15048. [PubMed: 19706469]

- Baba K, Sengupta A, Tosini M, Contreras-Alcantara S, Tosini G, 2010 Circadian regulation of the PERIOD 2::LUCIFERASE bioluminescence rhythm in the mouse retinal pigment epithelium-choroid. *Mol. Vis* 16, 2605–2611. [PubMed: 21151601]
- Baba K, Benleulmi-Chaachoua A, Journe A-S, Kamal M, Guillaume J-L, Dussaud S, Gbahou F, Yettou K, Liu C, Contreras-Alcantara S, Jockers R, Tosini G, 2013 Heteromeric MT₁/MT₂ melatonin receptors modulate photoreceptor function. *Science signaling* 6, ra89. [PubMed: 24106342]
- Baba K, DeBruyne JP, Tosini G, 2017 Dopamine 2 receptors activation entrains circadian clocks in mouse retinal pigment epithelium. *Sci. Rep* 7, 5103. [PubMed: 28698578]
- Besharse JC, Dunis DA, 1983 Methoxyindoles and photoreceptor metabolism: activation of rod shedding. *Science* 219, 1341–1343. [PubMed: 6828862]
- Bobu C, Hicks D, 2009 Regulation of retinal photoreceptor phagocytosis in a diurnal mammal by circadian clocks and ambient lighting. *Invest. Ophthalmol. Vis. Sci* 50, 3495–3502. [PubMed: 19234351]
- Bok D, 1993 The retinal pigment epithelium: a versatile partner in vision. *J Cell Sci* 17, 189–195.
- Fujieda H, Hamadanizadeh SA, Wankiewicz E, Pang SF, Brown GM, 1999 Expression of mtl melatonin receptor in rat retina: evidence for multiple cell targets for melatonin. *Neuroscience* 93, 793–799. [PubMed: 10465462]
- Gianesini C, Hiragaki S, Laurent V, Hicks D, Tosini G, 2016 Cone viability is affected by disruption of melatonin receptors signaling. *Invest. Ophthalmol. Vis. Sci* 57, 94–104. [PubMed: 26780313]
- Grace MS, Chiba A, Menaker M, 1999 Circadian control of photoreceptor outer segment membrane turnover in mice genetically incapable of melatonin synthesis. *Vis. Neurosci* 16, 909–918. [PubMed: 10580726]
- Hicks D, Molday RS, 1985 Localization of lectin receptors on bovine photoreceptor cells using dextran-gold markers. *Invest. Ophthalmol. Vis. Sci* 26, 1002–1013. [PubMed: 2409048]
- Hiragaki S, Baba K, Coulson E, Kunst S, Spessert R, Tosini G, 2014 Melatonin signaling modulates clock genes expression in the mouse retina. *PLoS One* 9, e106819. [PubMed: 25203735]
- Kunst S, Wolloscheck T, Kelleher DK, Wolfrum U, Sargsyan SA, Iuvone PM, Baba K, Tosini G, Spessert R, 2015 *Pgc-1α* and *Nr4a1* are target genes of circadian melatonin and dopamine release in murine retina. *Invest. Ophthalmol. Vis. Sci* 56, 6084–6094. [PubMed: 26393668]
- La Vail MM, 1976 Rod outer segment disk shedding in the rat retina: relationship to cyclic lighting. *Science* 194, 1071–1073. [PubMed: 982063]
- Liang FQ, Aleman TS, Zaixin Y, Cideciyan AV, Jacobson SG, Bennett J, 2001 Melatonin delays photoreceptor degeneration in the rds/rds mouse. *Neuro-report* 12, 1011–1014.
- Liu C, Fukuhara C, Wessel JH, Iuvone PM, Tosini G, 2004 Localization of *Aa-nat* mRNA in the rat retina by fluorescence in situ hybridization and laser capture microdissection. *Cell Tissue Res* 315, 197–201. [PubMed: 14618388]
- Milev NB, Reddy AB, 2015 Circadian redox oscillations and metabolism. *Trends Endocrinol. Metab* 26, 430–437. [PubMed: 26113283]
- Nandrot EF, Kim Y, Brodie SE, Huang X, Sheppard D, Finnemann SC, 2004 Loss of synchronized retinal phagocytosis and age-related blindness in mice lacking avB5 integrin. *J. Exp. Med* 200, 1536–1545.
- Nguyen-Legros J, Hicks D, 2000 Renewal of photoreceptor outer segments and their phagocytosis by retinal pigment epithelium. *Int. Rev. Cytol* 196, 245–313. [PubMed: 10730217]
- Rosen R, Hu DN, Perez V, Tai K, Yu GP, Chen M, Tone P, McCormick SA, Walsh J, 2009 Urinary 6-sulfatoxymelatonin level in age-related macular degeneration patients. *Mol. Vis* 15, 1673–1679. [PubMed: 19710945]
- Ruan G-X, Allen GC, Yamazaki S, McMahon DC, 2008 An autonomous circadian clock in the inner mouse retina regulated by Dopamine and Gaba. *PLOS Biol* 6, e249. [PubMed: 18959477]
- Ruggiero L, Connor MP, Chen J, Langen R, Finnemann SC, 2012 Diurnal, localized exposure of phosphatidylserine by rod outer segment tips in wildtype but not *Itgb5*^{-/-} or *Mfge8*^{-/-} mouse retina. *Proc. Natl. Acad. Sci. U. S. A* 109, 8145–8148. [PubMed: 22566632]

- Sengupta A, Baba K, Mazzoni F, Pozdeyev NV, Strettoi E, Iuvone PM, Tosini G, 2011 Localization of melatonin receptor 1 in mouse retina and its role in the circadian regulation of the electroretinogram and dopamine levels. *PLoS One* 6, e24483. [PubMed: 21915336]
- Teirstein PS, Goldman AI, O'Brien PJ, 1980 Evidence for both local and central regulation of rat rod outer segment disc shedding. *Invest. Ophthalmol. Vis. Sci* 19, 1268–1273. [PubMed: 7429763]
- Terman JS, Reme CE, Wirz-Justice A, 1993 Rod outer segment disk shedding in rats with lesions of the suprachiasmatic nucleus. *Brain Res* 605, 256–264. [PubMed: 8481775]
- Tosini G, Menaker M, 1996 Circadian rhythms in cultured mammalian retina. *Science* 272, 419–421. [PubMed: 8602533]
- Tosini G, Menaker M, 1998 The clock in the mouse retina: melatonin synthesis and photoreceptor degeneration. *Brain Res* 789, 221–228. [PubMed: 9573370]
- Tosini G, Davidson AJ, Fukuhara C, Kasamatsu M, Castanon-Cervantes O, 2007 Localization of a circadian clock in mammalian photoreceptors. *Faseb J* 21, 3866–3871. [PubMed: 17621597]
- Tosini G, Baba K, Hwang CK, Iuvone PM, 2012 Melatonin: an underappreciated player in retinal physiology and pathophysiology. *Exp. Eye Res* 2012 (103), 82–89.
- Tosini G, Owino S, Guillame J-L, Jockers R, 2014 Melatonin receptors: latest insights from mouse models. *BioEssays* 38, 778–787.
- White MP, Fisher LJ, 1989 Effects of exogenous melatonin on circadian disc shedding in the albino rat retina. *Vis. Res* 29, 167–179. [PubMed: 2800345]
- Wiechmann AF, 2002 Regulation of gene expression by melatonin: a microarray survey of the rat retina. *J. Pineal Res* 33, 178–185. [PubMed: 12220334]
- Wiechmann AF, Summers JA, 2008 Circadian rhythms in the eye: the physiological significance of melatonin receptors in ocular tissues. *Prog. Retin. Eye Res* 27, 137–160. [PubMed: 18316227]
- Xu XJ, Wang SM, Jin Y, Hu YT, Feng K, Ma ZZ, 2017 Melatonin delays photoreceptor degeneration in a mouse model of autosomal recessive retinitis pigmentosa. *J. Pineal Res* 10.1111/jpi.12428.
- Yi C, Pan X, Yan H, Guo M, Pierpaoli W, 2005 Effects of melatonin in age-related macular degeneration. *Ann N.Y. Acad. Sci* 1057, 384–392. [PubMed: 16399908]

Fig. 1.

Representative photomicrographs of the methodology used to count phagosomes in mouse RPE (A). DAPI staining allowed precise visualization of retinal-RPE morphology, while phagosome counting was rendered possible by rhodopsin immunodetection with anti-rhodopsin antibody Rho4D2. Daily rhythm in the number of phagosomes is measured in C3H-f^{+/+} mice (B) MT₁^{-/-} mice (C) and MT₂^{-/-} (D) mice. A daily rhythm in the number of phagosomes was present in all three genotypes (one-way ANOVA, $P < 0.01$, in all cases. The power of the performed test with $\alpha = 0.05$ was equal to 1.0 in all cases.). In C3H-f^{+/+} mice the peak in the number of phagosomes occurred at ZT1 (i.e., 1-hr after light onset) whereas in MT₁^{-/-} and MT₂^{-/-} mice the peak occurred 3 h earlier (ZT22), when the mice were still in darkness. Results are presented as mean $\pm$ S.E.M.

Fig. 2.

Photomicrographs of retinas from young (3–4 months) and aged (17–18 months) old C3H-f $^{+/+}$. (A) Young C3H-f $^{+/+}$ (B) young MT $_1^{-/-}$ (C) young MT $_2^{-/-}$ (D) old C3H-f $^{+/+}$ (E) old MT $_1^{-/-}$ and (F) old MT $_2^{-/-}$ mice. Similar data have been obtained in other mice (n = 3) for each genotype (see Baba et al., 2009 for details).

Fig. 3.

Cryosections (10 μm) of the posterior eye cup of young (3–4 months) and aged (17–18 months) old C3H- $f^{+/+}$. (A) Young C3H- $f^{+/+}$ (B) young $MT_1^{-/-}$ (C) young $MT_2^{-/-}$ (D) old C3H- $f^{+/+}$ (E) old $MT_1^{-/-}$ and (F) old $MT_2^{-/-}$ mice. Eye sections were examined under wide field fluorescent microscope exhibiting DAPI stained (cyan) nuclei of the RPE cells. RPE cells of old $MT_1^{-/-}$ and $MT_2^{-/-}$ mice contain more autofluorescent intracellular granules (detected in Rhodamine channel, red) than C3H- $f^{+/+}$. Bars in all figures are 10 μM . Similar data have been obtained in other mice ($n = 3$) for each genotype. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

COSINOR Analysis of the Phagocytic Activity by the RPE of C3H-f^{+/+} and MT₁^{-/-} and MT₂^{-/-} mice. A significant daily rhythm in phagocytic activity was present in all three genotypes ($P < 0.05$). A significant difference was present in the acrophase between C3H-f^{+/+} and MT₁^{-/-} and MT₂^{-/-} ($P < 0.05$). No differences were observed among the three genotypes in the mesor or in amplitude ($P > 0.05$).

	C3H-f ^{+/+}	MT ₁ ^{-/-}	MT ₂ ^{-/-}
Mesor	17.3 +/-1.3	19.2 +/-1.3	17.2 +/- 1.1
Amplitude	6.7 +/- 1.9	6.0 +/- 1.9	6.5 +/- 1.6
Acrophase	3.7* +/- 1.1	0.2 +/- 1.2	1.3 +/- 0.9