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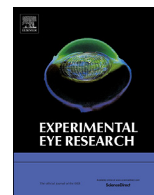

Melatonin signaling affects the timing in the daily rhythm of phagocytic activity by the retinal pigment epithelium

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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 (MT_1) or type 2 (MT_2) in a melatonin-proficient background and have shown that removal of MT_1 and MT_2 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 MT_1 and MT_2 knock-out mice. Our data indicate that in MT_1 and MT_2 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 MT_1 and MT_2 knock-out mice showed a significant increase in lipofuscin accumulation in the retinal pigment epithelium.

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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; 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 MT_1 and MT_2 (Tosini et al., 2014). Both of these receptors have been identified in the mouse retina. MT_1 receptors are present in photoreceptors, inner retinal neurons and retinal ganglion cells (Baba et al., 2009; Sengupta et al., 2011), whereas MT_2 receptors are present in photoreceptors and inner retinal neurons, but not in

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retinal ganglion cells (Baba et al., 2013). In rat, MT_1 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 synthetizing substantial quantities of melatonin (Grace et al., 1999).

Melatonin proficient-mice (C3H-$f^{+/+}$) and C3H-$f^{+/+}$ in which MT_1 (C3H/$f^{+/+}MT_1^{-/-}$) and MT_2 (C3H/$f^{+/+}MT_2^{-/-}$) 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-$f^{+/+}$-$MT_1^{-/-}$ (MT_1) or C3H-$f^{+/+}$-$MT_2^{-/-}$ ($MT_2^{-/-}$) 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 μm 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 μm on which phagosomes (defined as immunofluorescent inclusions of >1 μm – see (Bobu and Hicks, 2009 for details) were manually identified and counted. Data presented are the means of the no. of phagosomes per 150 μm 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+/- 1.669 (SEM); $MT_1^{-/-}$ = 18.47 +/- 1.42; $MT_2^{-/-}$ = 18.17 +/- 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 $MT_1^{-/-}$ (E) 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 (αvβ5 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*

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