Brief Communication

Classical Photoreceptors Regulate *Melanopsin* mRNA Levels in the Rat Retina

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Recent studies have demonstrated that melanopsin is a key photopigment in the mammalian circadian system. This novel opsin is exclusively expressed in retinal ganglion cells that are intrinsically sensitive to light, perhaps responding via a melanopsin-based signaling pathway. Previous investigations using transgenic mice have also demonstrated that ablation of the classical photoreceptors and of melanopsin prevents entrainment of several circadian rhythms, thus demonstrating that these photoreceptors are necessary and sufficient for circadian photoreception. In this study, we investigated the effect of photoreceptor degeneration on *melanopsin* mRNA regulation in RCS/N-*rdy* rats (Royal College of Surgeons rats with a defect in the retinal dystrophy gene). We used animals at postnatal day 21 (P21), P33, P45, and P60. At P60 degeneration of the retina in RCS/N-*rdy* has advanced to the point where the majority of the photoreceptors have degenerated. Our data indicate that *melanopsin* mRNA levels were rhythmic in light/dark cycle and in constant darkness in congenic controls (RCS/N-*rdy*$^+$) and in RCS/N-*rdy* at P21 (i.e., before the degeneration of the photoreceptors). On the other hand, in RCS/N-*rdy* at P60, *melanopsin* mRNA levels were greatly reduced (<90%) and not rhythmic. Photoreceptor degeneration did not affect the expression of *pituitary adenylate cyclase-activating polypeptide* mRNA (a marker for melanopsin-containing ganglion cells). Our results suggest that classical photoreceptors (rods and cones) regulate the expression of *melanopsin* mRNA in the rat. Because RCS/N-*rdy* rats are a model for studies on retinitis pigmentosa in human, our data may provide an important insight on melanopsin function in patients affected by retinitis pigmentosa.

Key words: melanopsin; circadian rhythm; RCS rat; circadian photoreception; retina; photoreceptor degeneration

Introduction

The study of the photoreceptors and photopigments that mediate circadian photoreception in mammals is emerging as a new and exciting aspect of retinal neurobiology. A series of elegant studies in the 1990s demonstrated that classical photoreceptors (rods and cones) are not necessary for the entrainment of circadian rhythms in mammals (Foster et al., 1991; Freedman et al., 1999; Lucas et al., 1999), thus indicating that an undiscovered photoreceptor (photopigment) in the mammalian retina is responsible for the photoentrainment of circadian rhythms.

The most likely candidate to emerge as the circadian retinal photopigment is a mammalian homolog of *Xenopus* melanopsin (Provencio et al., 1998). In mammals, *melanopsin* mRNA (and protein) is only expressed in a small population of retinal ganglion cells (RGCs) (Provencio et al., 2000) that are directly photosensitive (Berson et al., 2002), expresses pituitary adenylate cyclase-activating polypeptide (PACAP) (Hannibal et al., 2002), and gives origin to the retinohypothalamic tract (Gooley et al., 2001; Hattar et al., 2002). Additional studies have shown that

melanopsin (or *Opn4*) plays an important role in the complex system that regulates circadian photoreception (Panda et al., 2002; Ruby et al., 2002; Hattar et al., 2003), sleep–wake states (Gooley et al., 2003), and the pupillary light reflex (Lucas et al., 2003).

New experimental evidence has demonstrated that melanopsin-expressing RGCs may receive input from rod and/or cone photoreceptors (Belenky et al., 2003), thus suggesting that some interaction may exist between the classical photoreceptors and this newly discovered photoreceptive system. However, a previous study has reported that, in mice lacking rods and cones, melanopsin regulation was not affected by the loss of the photoreceptors (Semo et al., 2003).

Retinitis pigmentosa (RP) is one of the leading causes of blindness in humans (Kaplan et al., 1990). Although RP is most commonly caused by defects intrinsic to photoreceptors, several cases have been identified with recessive mutations of the receptor tyrosine kinase *Mertk* gene (Li et al., 2000). A recent paper has reported that the gene encoding for *Mertk* corresponds to the retinal dystrophy (*rdy*) locus of the RCS (Royal College of Surgeons) rat (D'Cruz et al., 2000). In this animal, which is the most studied of the available animal models of retinal dystrophy, the gene defect renders cells of the retinal pigment epithelium unable to phagocytose and shed photoreceptor outer segments at a normal rate (Dowling and Sidman, 1962). This inability leads to photoreceptor cell death.

Therefore, RCS/N-*rdy* rats represent a useful model to investigate the effect of photoreceptor degeneration on melanopsin

expression and to understand the possible function(s) of melanopsin in humans affected by RP.

The aim of this study was to investigate the pattern of expression of *melanopsin* mRNA under different photic conditions and the effect that photoreceptor degeneration has on *melanopsin* mRNA regulation in RCS/N-*rdy* rats. Our data indicate that photoreceptor degeneration induced the loss of photic and circadian control of *melanopsin* mRNA, and *melanopsin* transcripts were dramatically reduced in retinas lacking photoreceptor cells. The reduction in *melanopsin* transcripts is not attributable to the loss (death) of melanopsin-positive RCGs but rather to a downregulation of *melanopsin* mRNA transcription within these cells. These data indicated that classical photoreceptors are required for *melanopsin* mRNA expression.

Materials and Methods

Animals and tissue collection. Tan-hooded RCS/N-*rdy*+ rats homozygous for the normal *rdy* allele and congenic age-matched RCS/N-*rdy* homozygotes with retinal dystrophy were used in this study. Animals were raised from birth at Morehouse School of Medicine in a 12 hr light/dark (LD) cycle of illumination with lights (250 lux) on from Zeitgeber time 0 (ZT0) to ZT12. Food and water were available *ad libitum*. We used animals at postnatal day 21 (P21), P33, P45, and P60. At P60, degeneration of the retina in RCS/N-*rdy* has advanced to the point at which the majority of the photoreceptors have degenerated (Dowling and Sidman, 1962; Doyle et al., 2002).

To investigate the expression of melanopsin in LD cycles, animals were killed at ZT2, ZT6, ZT10, ZT14, ZT18, and ZT22. When animals were killed during the night, the procedure was performed in dim red light ($<$ 1 lux). To investigate the pattern of expression in constant darkness (DD), rats were transferred into constant darkness for 2 d before being killed. Samples were then collected on the third day at circadian time 2 (CT2), CT6, CT10, CT14, CT18, and CT22. Retinas were dissected, immediately frozen on dry ice, and stored at −80°C.

All procedures were approved by the Animal Care and Use Committee and conformed to National Institutes of Health guidelines.

Real-time quantitative reverse transcription-PCR. Total RNA was isolated form each retina using TRIZOL reagent (Invitrogen, Grand Island, NY) after sonication. RNA was treated with DNase I to remove any traces of genomic DNA. First-strand cDNA was synthesized from 1 μg of each RNA sample using oligo-dT and Omniscript reverse transcriptase (Qiagen, Valencia, CA) according to the protocol of the manufacturer. Each set of samples was simultaneously processed for RNA extraction, DNase I treatment, cDNA synthesis, and PCR. Real-time quantitative reverse transcription (RT)-PCR was performed with SYBR Green using *i*Cycler (Bio-Rad, Hercules, CA). Briefly, primers used were as follows: for rat *melanopsin* (GenBank accession number AY072689), forward, 5′-atctggtgatcacacgtcca-3′ and reverse, 5′-tagtcccaggagcaggatgt-3′ (169 bp product, spanning a ∼600 bp intron); for rat *PACAP* (GenBank accession number M63006), forward, 5′-acagcgtctcctgttcacct-3′ and reverse, 5′-cctgtcggctgggtagtaaa-3′ (179 bp product, spanning a ∼2000 bp intron); for rat *rhodopsin* (GenBank accession number Z46957), forward, 5′-gcagtgttcatgtgggattg-3′ and reverse, 5′-ctgccttctgagtggtagcc-3′ (191 bp product); for rat *blue cone opsin* (GenBank accession number AF051163), forward, 5′-gtaccacattgctcccgtct-3′ and reverse, 5′-agacctgctacagagcccaa-3′ (282 bp product); for rat *green cone opsin* (GenBank accession number AF031528), forward, 5′-tcatttcctgggagagatgg-3′ and reverse, 5′-ttttctgttgcttgccactg-3′ (326 bp product); and for rat *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) (GenBank accession number X02231), forward, 5′-agacagccgcatcttcttgt-3′ and reverse, 5′-tgatggcaacaatgtccact-3′ (142 bp product). Cycling parameters were 95°C for 8 min from activate *Taq*DNA polymerase, 40 cycles of 95°C for 15 sec, 60°C for 45 sec, and 72°C for 30 sec. The fluorescence of the accumulating product was measured after each cycle at 88°C, 89°C, 86°C, 86°C, 87°C, and 85°C (i.e., 4°C below the product temperature) for *melanopsin*, *PACAP*, *rhodopsin*, *blue cone opsin*, *green cone opsin*, and *Gapdh* mRNA, respectively. To confirm the specificity of PCR products, melting

curves were determined using *i*Cycler software, and samples were run on an agarose gel. The amount of amplified product was calculated from plasmid standards. Expression levels of each transcript were normalized by comparison with the amount of *Gapdh* mRNA

Immunohistochemistry and Western blot. Eyeballs obtained from RCS/N-*rdy*+ and RCS/N-*rdy* animals at P60 killed at ZT14 were punctured and then fixed with 4% paraformaldehyde in PBS, pH 7.0, for 6 hr at 4°C. The eyes were transferred to a solution of 30% sucrose for 12–14 hr, embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN), cut into 12- to 16-μm-thick cryosections, and stored at −80°C until processed for immunohistochemistry using a procedure described by Hannibal et al. (2002). A well characterized rabbit anti-melanopsin antiserum (code 41K9; generous gift from Dr. Jens Hannibal, University of Copenhagen, Copenhagen, Denmark) diluted 1:20,000 combined with tyramide amplication was used. Slides were mounted and then viewed with a Zeiss (Oberkochen, Germany) Axioskop microscope equipped with epifluorescence. The Western blot was performed as described by Hannibal et al. (2002) using the same antibody (code 41K9) diluted 1:1000. Both the immunohistochemistry and Western blot were repeated at least three times with independent samples.

Results
Effect of photoreceptor degeneration on melanopsin regulation
To investigate the effects of photoreceptor degeneration on melanopsin, we measured *melanopsin* mRNA levels in RCS rats at P60 (Fig. 1*A*). In RCS/N-*rdy*+ (controls), *melanopsin* transcripts showed a high-amplitude rhythm in LD and in DD (ANOVA; $p < 0.001$, in both cases), with high levels during early night (ZT10–ZT14) relative to the early day (ZT22–ZT2). Thus, we can conclude that, in RCS/N-*rdy*+ (controls), *melanopsin* mRNA expression is controlled by the circadian clock. Surprisingly, in animals lacking photoreceptors (RCS/N-*rdy*), *melanopsin* mRNA levels were significantly reduced (∼90%), and no rhythmic expression was observed (ANOVA; $p > 0.33$) (Fig. 1*A*).

Melanopsin-immunoreactive cells were observed in the ganglion cell layer of RCS/N-*rdy*+ (Fig. 1*B*) but not in RCS/N-*rdy* (Fig. 1*C*). The distribution pattern of melanopsin immunoreactivity in RCS/N-*rdy*+ animals was similar to that described in previous studies (Hannibal et al., 2002; Hattar et al., 2002). In addition to immunohistochemistry, expression of melanopsin protein was confirmed by Western blotting in RCS/N-*rdy*+ but not in RCS/N-*rdy* rat (Fig. 1*D*). In RCS/N-*rdy*+ melanopsin seems to be more abundant at ZT14 than at ZT2 (Fig. 1*D*).

The mutation in the *Mertk* gene is not directly responsible for the results obtained in RCS/N-*rdy*
To test whether the change in *melanopsin* mRNA expression was a consequence of the mutation in the *Mertk* gene or the result of photoreceptor degeneration, we investigated the pattern of expression of *melanopsin* mRNA in RCS/N-*rdy* rats at P21 (i.e., before the photoreceptors start to degenerate). *Melanopsin* transcripts were clearly rhythmic in RCS/N-*rdy* rats at P21 (ANOVA; $p < 0.01$) (Fig. 2), with overall levels that were not significantly different from what was observed in RCS/N-*rdy*+ of the same age (*t* tests; $p > 0.1$ in all cases) (Fig. 2). The pattern of expression of *melanopsin* mRNA was also similar among RCS/N-*rdy* at P21 and RCS/N-*rdy*+ at P21 and P60 (Figs. 1*A*, 2).

PACAP mRNA is not affected by photoreceptor degeneration
Previous studies have shown that, although RCS/N-*rdy* mutants undergo a process that leads to the loss of the photoreceptors, the RGCs are not affected, and, indeed, we found that *Thy-1* mRNA levels (a marker for ganglion cells) were not significantly different between RCS/N-*rdy* and RCS/N-*rdy*+ rats at P60 (*t* test; $p > 0.1$)
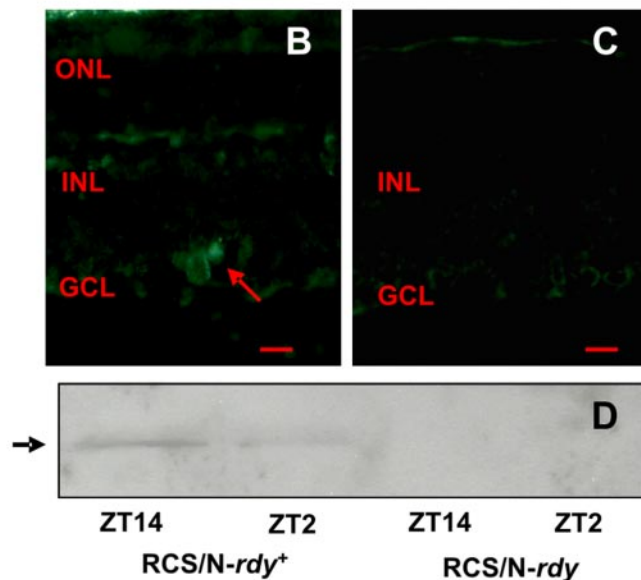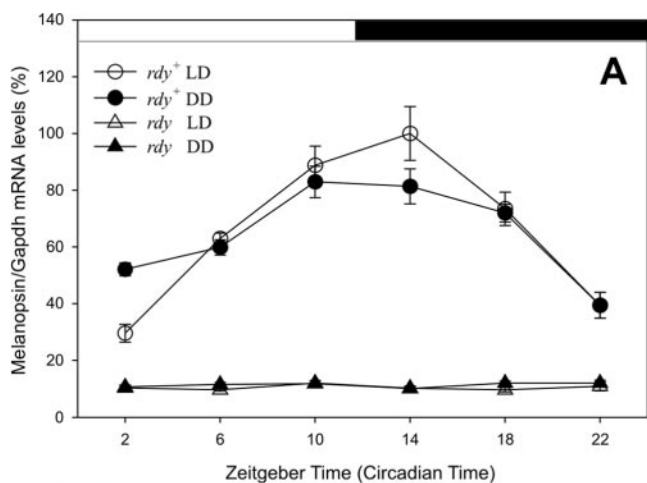
Figure 1. *A*, *Melanopsin* mRNA levels in RCS/N-*rdy* and RCS/N-*rdy*⁺ retina at P60. *Melanopsin* mRNA levels were measured using real-time quantitative RT-PCR. mRNA levels were rhythmic in LD and DD (ANOVA; $p < 0.001$) in RCS/N-*rdy*⁺ animals but not in RCS/N-*rdy* (ANOVA; $p > 0.1$). Data are expressed as mean ± SEM ($n \geq 4$ retinas). All values were normalized with respect to the values obtained in RCS/N-*rdy*⁺ animals at ZT14. The white bar at the top of the graph represents the period of light (ZT0–ZT12), and the black bar represents the period of darkness (ZT12–ZT24) when the rats were exposed to LD cycles. Photomicrographs showing the expression pattern of melanopsin-immunoreactive cells (arrow) in a 60-d-old RCS/N-*rdy*⁺ rat with a normal retina (*B*) and in a 60-d-old RCS/N-*rdy* (*C*). Scale bars, 20 μm. ONL, Outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. In the dystrophic retina, the photoreceptor layer is absent. *D*, Western blot of protein extracts from pooled retinas probed with the melanopsin antibody. Immunoreactivity (as a 50 kDa band, arrow) was present in RCS/N-*rdy*⁺ at ZT14, and a weaker band was also present at ZT2. No immunoreactivity was detected in RCS/N-*rdy* rats.

Figure 2. *Melanopsin* mRNA levels in RCS/N-*rdy* at P21. All values were normalized with respect to the values obtained at ZT14 in RCS/N-*rdy*⁺. The white bar at the top of the graph represents the period of light (ZT0–ZT12), and the black bar represents the period of darkness (ZT12–ZT24) when the rats were exposed to LD cycles. Data are expressed as mean ± SEM ($n \geq 4$ retinas).

Figure 3. *PACAP* mRNA levels in RCS/N-*rdy*⁺ (white bars) and RCS/N-*rdy* (black bars) retinas at P60 at ZT2 and ZT14. Data are expressed as mean ± SEM ($n \geq 4$ retinas). *PACAP* mRNA levels did not differ between RCS/N-*rdy*⁺ and RCS/N-*rdy* at both time points ($p > 0.05$ in all cases).

(data not shown). However, it must be noted that *melanopsin* is only expressed in a small subpopulation (1–2%) of RGCs, and, therefore, small changes in *Thy-1* mRNA levels that may result as a consequence of the loss of a few melanopsin-positive RGCs may be difficult to detect. A previous study has reported that *melanopsin* mRNA and *PACAP* mRNA are coexpressed in the same RGCs. Therefore, we decided to investigate the effects of photoreceptor degeneration on the levels of *PACAP* mRNA in RCS/N-*rdy* to test whether the observed changes in *melanopsin* mRNA were attributable to the loss (death) of a subpopulation of RGCs or to a change in the regulation of *melanopsin* transcription
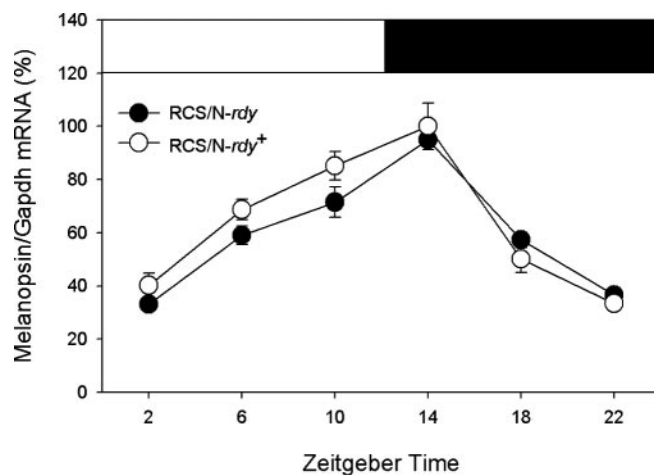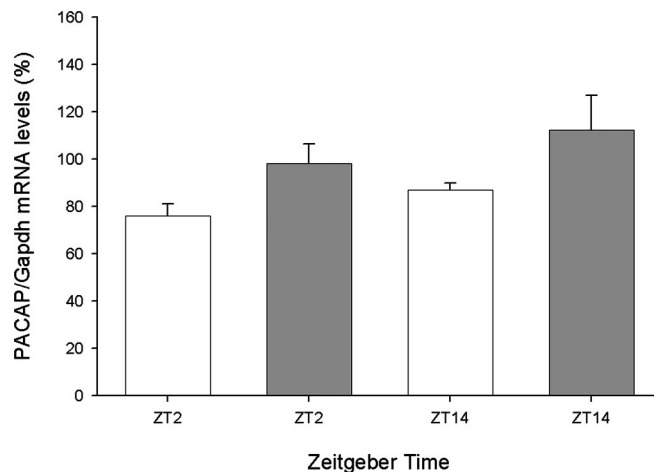
within these cells. Surprisingly, we observed that *PACAP* transcripts were not reduced by the loss of photoreceptors (*t* tests; $p > 0.1$) (Fig. 3), thus demonstrating that the observed reduction in *melanopsin* mRNA is not attributable to the loss of melanopsin-positive RGCs but rather to the change in the synthesis of this putative photopigment that follows the degeneration of the photoreceptor cells.

Photoreceptor loss affects *melanopsin* mRNA levels
In our last experiment, we investigated the effect of photoreceptor degeneration on the levels and pattern of expression of *melanopsin* mRNA in RCS-*rdy* rats at P21, P33, P45, and P60. *Melanopsin* mRNA levels showed a steady decrease between P21 and P60 (*t* tests; $p < 0.01$ in all cases) (Fig. 4*A*). As expected, *rhodopsin* transcripts showed a dramatic reduction with the progression of photoreceptor loss (Fig. 4*B*). The greatest reduction in the level of *rhodopsin* mRNA was observed between P33 and P45. *Green cone opsin* (Fig. 4*C*) and *blue cone opsin* (Fig. 4*D*) transcripts showed a significant decrease between P21 and P60 (*t* test; $p <$
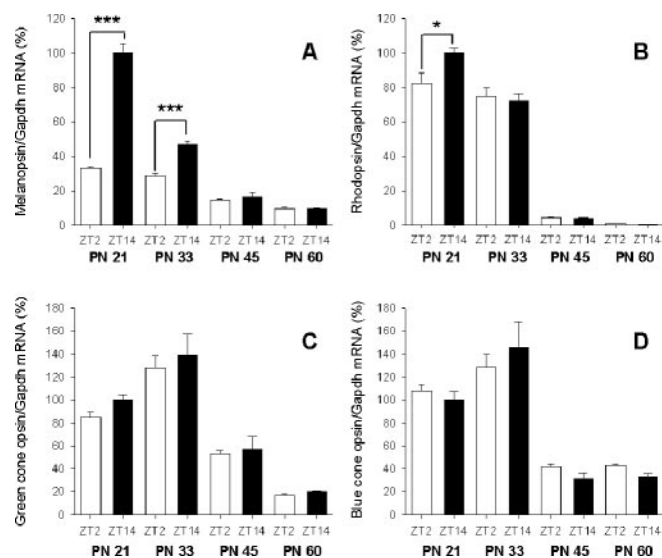
Figure 4. Changes in photopigments mRNA in RCS/N-*rdy* rats at P21, P33, P45, and P60. *A, Melanopsin; B, rhodopsin; C, green cone opsin;* and *D, blue cone opsin.* mRNA levels were measured at ZT2 (white bars) and ZT14 (black bars). These time points correspond to the trough and the peak of the rhythm for *melanopsin, rhodopsin,* and *green cone opsin* transcripts, whereas *blue cone opsin* mRNA was found not rhythmic (K. Sakamoto and G. Tosini, unpublished observations). Data are expressed as mean ± SEM ($n \geq 3$ retinas). *$p < 0.05$; ***$p < 0.001$. PN, Postnatal day.

0.01 in all cases), but the reduction was not as dramatic as observed in *rhodopsin* mRNA (Fig. 4).

Interestingly, *melanopsin* mRNA rhythmicity disappeared between P33 and P45 (i.e., in parallel with the reduction in *rhodopsin* mRNA) (Fig. 4*A,B*).

Discussion

The focus of this study was to investigate the effects of photoreceptor degeneration on the regulation of *melanopsin* mRNA in the rat retina. Our data demonstrated that, in animals lacking photoreceptors, *melanopsin* mRNA transcription is arrhythmic, and melanopsin protein is not detectable (Fig. 1*A–D*). In addition, we also demonstrated that the observed changes in *melanopsin* mRNA regulation are an outcome of photoreceptor degeneration rather than a direct effect of the *Mertk* mutation in RCS/N-*rdy* rats (Figs. 2, 4). Finally, our results indicate that the reduction in *melanopsin* mRNA is not attributable to the loss of RGCs because *Thy-1* mRNA and, more importantly, *PACAP* mRNA levels are not affected by the reduction in the photoreceptor number (Fig. 3).

Although several studies have reported the distribution of *melanopsin* mRNA and protein (Provencio et al., 2000; Gooley et al., 2001; Hattar et al., 2002), we found that only one paper has suggested a possible day–night difference in melanopsin expression (Hannibal et al., 2002). The data presented here agree with those preliminary results and clearly demonstrate that *melanopsin* mRNA levels are rhythmic in LD and DD, with higher levels at ZT (CT) 14 and lowest at ZT (CT) 2. Moreover, our data show that *melanopsin* transcripts are not affected by illumination once the photoreceptors have degenerated (Figs. 1*A*, 4). Such a result suggests that *melanopsin* mRNA levels in the RCS/N-*rdy* at P60 do not generate enough melanopsin protein that would be required to successfully perform the normal phototransduction processes.

A recent study has reported that the gene encoding for the receptor tyrosine kinase (*Mertk*) corresponds to the retinal dys-

trophy (*rdy*) locus of the RCS rat (D'Cruz et al., 2000). The finding that RCS/N-*rdy* rats at P21 showed a clear rhythm in *melanopsin* mRNA, which was not different from what was observed in control animals (Fig. 2), together with the fact that *melanopsin* and *Mertk* transcripts are not expressed in the same type of retinal cells (D'Cruz et al., 2000; Provencio et al., 2000) demonstrated that the results obtained are not attributable to the direct effect of the mutation in *Mertk* gene. Hence, we believe the observed results could be an indirect consequence of (or mediated by) photoreceptor degeneration.

Previous investigations have reported that photoreceptor degeneration in RCS/N-*rdy* rat does not affect the ganglion cell layer (Villegas-Perez et al., 1998). Our data confirm and expand those data in demonstrating that *Thy-1* mRNA [a marker for RGCs (Barnstable and Drager, 1984)] and, more importantly, *PACAP* mRNA levels are not reduced in rats lacking photoreceptors. Because previous studies have shown that *PACAP* and *melanopsin* transcripts in the rat retina are coexpressed and have identical distribution (Hannibal et al., 2002), we can conclude that the observed reduction in *melanopsin* mRNA levels at P60 was not attributable to the loss (death) of melanopsin-positive RCGs but rather to a downregulation of *melanopsin* mRNA transcription within these cells.

The rat retina contains rod and cone (green and blue) photoreceptors. Rods represent ~99% of the photoreceptors, and green cones represent 80–85% of all the cones (Szel and Rohlich, 1992). Our data show that the reduction in *rhodopsin* mRNA levels progresses with age, and, by P60, <1% of *rhodopsin* mRNA is found in RCS/N-*rdy* retinas (Fig. 4*B*). In addition, our results show that *melanopsin* mRNA became arrhythmic between P33 and P45, when a dramatic decrease in *rhodopsin* mRNA was observed but when *cone opsin* transcripts are still relatively abundant (Fig. 4*C,D*). Such a result suggests that rod photoreceptors may be the key players in controlling *melanopsin* mRNA in rat retina.

As we have mentioned previously, in mice lacking rods and cones, *melanopsin* mRNA levels were not affected by the loss of photoreceptors (Semo et al., 2003), thus showing a dramatic difference from the result obtained in our study. We believe that there are at least two possible explanations for such a discrepancy. First, it is plausible that the difference in the results obtained between the two studies is attributable to the fact that two different species (rat and mouse) were used. Alternatively, the observed discrepancy can be attributed to the fact that the different mechanisms that lead to photoreceptor degeneration in the rodless–coneless mouse and in the RCS/N-*rdy* rat may affect melanopsin regulation in a different manner. In that regard, it may be significant to mention that, in some blind subjects, with no conscious perception of light, the photic input to the circadian system is preserved (Czeisler et al., 1995; Klerman et al., 2002). Such a result indicates that different modalities leading to the degeneration of the classical photoreceptors may have different effects on the photoreceptive system mediating circadian photoreception.

Although the data presented here do not question the role of melanopsin as an important component in the pathway involved in circadian photoreception (Panda et al., 2002, 2003; Ruby et al., 2002; Hattar et al., 2003), the finding that *melanopsin* mRNA transcription is greatly affected by the loss of rod and cone photoreceptors indicates that, at least in the rat, *melanopsin* mRNA regulation is somehow controlled by the classical photoreceptors.

Finally, we want to mention that melanopsin is believed to be involved in the mediation of several aspects of nonvisual photoreception that may affect human health (Gooley et al., 2003). As

we have mentioned previously, RCS/N-*rdy* rats represent a well established model to study RP in human, and our new findings suggest that RP may also affect melanopsin-based photoreception and thus the processes that are mediated by this photopigment.

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