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Effect of photoreceptor degeneration on circadian photoreception and free-running period in the Royal College of Surgeons Rat

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Abstract

The study of how the retina processes the photic information required for the entrainment of the circadian system is an exciting new topic in retinal neurobiology. We have recently shown that in RCS/N-*rdy* rats *melanopsin* mRNA levels are dramatically reduced (about 90 %) and melanopsin immunoreactivity cannot be detected in the retina of these rats at sixty days of age. Although RCS/N-*rdy* rats are a widely-used model to investigate mechanisms of photoreceptor degeneration, no study has investigated circadian photoreception in these animals. The aim of this study was to examine circadian photoreception in RCS/N-*rdy*⁺ (*rdy*⁺) rats homozygous for the normal *rdy* allele and age-matched RCS/N-*rdy* (*rdy*) homozygotes with retinal dystrophy. No differences between RCS/N-*rdy* and *rdy*⁺ were observed in light-induced phase shift of locomotor activity at the three light intensities used (1×10^{-3} , 1×10^{-1} , and $1 \times 10^1 \mu\text{Wcm}^{-2}$). Surprisingly, we observed that in RCS/N-*rdy* the free-running period of the circadian rhythm of locomotor activity was shorter ($P < 0.01$) than in *rdy*⁺, thus suggesting that photoreceptor degeneration may affect the free-running period of the locomotor activity rhythm.

Classification terms

Sensory and motor system

Keywords

RCS rat; circadian rhythms; melanopsin; circadian photoreception; *retinitis pigmentosa*; MERTK

1. Introduction

The study of how the retina processes the photic information required for the entrainment of the circadian system is an exciting new topic in retinal neurobiology. It is now believed that while all effects of light on the circadian visual system are accounted for by three types of photoreceptors (i.e., rods, cones and intrinsically photosensitive retinal ganglion cells [*ipRGCs*]) (Berson et al., 2002; Hattar et al., 2002; Hattar et al., 2003; Panda et al., 2003) none of these photoreceptors is essential for entrainment. Indeed, the amplitude of the phase shifts in the locomotor activity rhythms in response to a light stimulus is not affected by the loss of the rods (Foster et al., 1991) or by the loss of rods and cones (Freedman et al., 1999). Removal

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of melanopsin only attenuates (40%) the ability of mice to phase-shift in response to a light stimulus (Panda et al., 2002; Ruby et al., 2002). Therefore, these data suggest that all the retinal photoreceptors contribute to the entrainment of the circadian rhythms and likely classical photoreceptors and retinal circuitry may modulate the photic response of *ipRGCs* (Belenky et al., 2003; Dacey et al., 2005; Sakamoto et al., 2004a; Sakamoto et al., 2005).

Further studies have also shown that melanopsin is involved in regulation of others non-image forming (NIF) visual functions such as masking and pupillary constriction in response to photic stimuli (Lucas et al., 2001; Lucas et al., 2003; Panda et al., 2003).

We have recently reported that in the Royal College Surgeons (RCS) rats homozygous for retinal dystrophy (*rdy*) at the post-natal day (PN) 60 melanopsin levels are dramatically (more than 90%) down-regulated once the classical photoreceptors have degenerated (Sakamoto et al., 2004a). The reduction of melanopsin levels is not due to the loss of the *ipRGCs* since PACAP (a marker for the *ipRGCs*) levels are not affected (Sakamoto et al., 2004a).

Although RCS/N-*rdy* rats are one of the most widely-used model to investigate mechanism of photoreceptors degeneration (Cuenca et al., 2005) no study has investigated the circadian visual system in these animals. Such a study may be particularly important since several cases of *retinitis pigmentosa* (RP) in humans have been identified with recessive mutations of the gene encoding receptor tyrosine kinase *Mertk* (Gal et al., 2000; Li et al., 2000) that correspond to the retinal dystrophy (*rdy*) locus of the RCS rat (D'Cruz et al., 2000). Therefore, the results obtained with this animal model may be used to predict how the circadian visual system is functioning in patients affected by this type of RP.

In the present study, we investigated circadian photoreception in RCS rats. Our experiments show that in the RCS rats the degeneration of the photoreceptors does not affect the capability to phase-shift in response to a light pulse of different intensities. Surprisingly, we discovered that photoreceptor degeneration altered the free-running period of the locomotor activity rhythm.

2. Results

2.1. Phase-shift of locomotor activity in *rdy* and *rdy*⁺

To characterize the circadian visual system in RCS rats, *rdy* and *rdy*⁺ rats were placed in a running wheel at the postnatal age of 40 days, entrained to LD cycles for five days and then expose to DD for 14 days. At PN 60 (+/- 2 days) animals were subjected to a 15 min pulse of light at ZT 15 and then returned to DD for an additional 10-14 days (Figure 1 A, B). *rdy* and *rdy*⁺ were tested at three different irradiances (1×10^{-3} , 1×10^{-1} and $1 \times 10^1 \mu\text{Wcm}^{-2}$). The circadian rhythm of locomotor activity was not phase-shifted by a 15 min pulse at the lowest irradiance level ($1 \times 10^{-3} \mu\text{Wcm}^{-2}$), whereas significant phase-shifts were observed with the other two irradiances (1×10^{-1} and $1 \times 10^1 \mu\text{Wcm}^{-2}$). In *rdy*⁺ rats the mean phase-shift (Figure 1 C) at $1 \times 10^{-1} \text{ mWcm}^{-2}$ was 156.2 min (N = 8; SEM = 29.349) whereas in *rdy* was 88.7 (N = 8; SEM = 12.419). At $1 \times 10^1 \text{ mWcm}^{-2}$ the mean phase-shift in *rdy* was 212.5 min (N = 10; SEM = 18.139) and in *rdy*⁺ was 160.0 (N = 7; SEM = 11.339). Although, *rdy* rats showed a larger phase-shift than *rdy*⁺, no significant difference in the amplitude of the phase between *rdy* and *rdy*⁺ was observed (*t*-test, $P > 0.1$, Figure 1 C). Interestingly, the amplitude of phase-shift in *rdy*⁺ was significantly greater at $1 \times 10^1 \text{ mWcm}^{-2}$ than at $1 \times 10^{-1} \text{ mWcm}^{-2}$ (*t*-test, $P < 0.01$), whereas no significant difference was observed in the *rdy* (*t*-test, $P > 0.1$, Figure 1 C).

2.2. The free running period of the locomotor activity rhythm (τ) in *rdy* and *rdy*⁺

During the course of our phase-shift experiment, we noticed that *rdy* rats free-ran with a τ shorter than 24-h (Figure 2 A), whereas *rdy*⁺ free-ran with a τ longer than 24-h (Figure 2 B).

The difference (about 1-h) between the free-running periods of the *rdy* (mean = 23.4-h, SEM = 0.031, N = 21) and *rdy*⁺ (mean = 24.2-h, SEM = 0.060, N = 18) was statistically significant (t-test, $P < 0.001$; Figure 2 C) and no overlap of τ was observed between the two genotypes (Figure 2 D). As we have already mentioned, a previous study has identified that the *Mertk* is the gene responsible for the *rdy* phenotype. Therefore, we wondered if the change in τ observed in the *rdy* was the results of an interaction between *Mertk* and the molecular mechanism that generates the circadian oscillation in the suprachiasmatic nucleus (SCN) of the hypothalamus. Hence, we decided to investigate if *Mertk* transcripts were present in the SCN. Using *in situ* hybridization, we were able to detect *Mertk* mRNA in the SCN (Figure 3 A, B), though its levels did not show any significant rhythmicity (ANOVA, $P > 0.1$; Figure 3 C).

2.3. Effect of enucleation on τ in *rdy* and *rdy*⁺

In our last experiment, we decided to investigate if bilateral enucleation had an effect on the free-running period of *rdy*⁺ rats. *rdy* and *rdy*⁺ rats were enucleated at PN 21 (i.e. before photoreceptors begin to degenerate) or at PN 40. Then, at PN 45, were placed in running wheels to monitor the running wheel activity. Our data (Figure 4 A, C) show that enucleation of *rdy*⁺ at PN 21 induced a significant change in the free-running period (mean = 23.3-h, SEM = 0.032, N = 5) with respect to what was observed in intact *rdy*⁺ (mean = 24.3-h, SEM = 0.027, N = 18). However, the free-running period of enucleated *rdy*⁺ (PN 21) was not different from the free-running period of the *rdy* (mean = 23.4-h, SEM = 0.031, N = 21; Figure 4 A, C). Enucleation of *rdy*⁺ at PN 40 did not induce any changes in τ (mean = 24.3-h, SEM = 0.058, N = 5, t-test, $P < 0.1$; Figure 4 C). Enucleation of *rdy* at PN 21 or PN 40 did not induce any significant change in the free-running period (t-test, $P > 0.1$ in all cases; Figure 4 B, C).

3. Discussion

Three different photoreceptors have been identified in the rat retina. The rods represent the vast majority of the classical photoreceptors while the cones (green and blue) represent only 2 % (Szel and Rohlich, 1992). The *ipRGCs* constitute about 1-2% of the total number of ganglion cells (Hannibal et al., 2002). Although the cones represent only a small number of the photoreceptors experimental evidence indicates that primarily 510 nm green and secondarily 375 nm near-ultraviolet cones may provide photic information to the rat SCN (Aggelopoulos and Meissl, 2000).

Our data demonstrate that although RCS/N-*rdy* rats have a massive reduction in the photoreceptive apparatus their capability to phase-shift in response to a pulse of light is not affected. However, our results also indicate that the free-running period in *rdy* is significantly shorter than in *rdy*⁺ rats.

The results of the phase-shift experiments are consistent with the data obtained in the *rd/rd* mouse (Foster et al., 1991; Provencio and Foster, 1995; Yoshimura and Ebihara, 1996) and in the *rdta* (Lupi et al., 1999) and *rdta/cl* (Freedman, 1999) since they show that the degeneration of photoreceptor does not affect the capability of the animals to phase shift. However, it must be noticed that an important difference exists between our results and those obtained in the mouse, since in the *rd* mouse melanopsin levels are unaffected by the degeneration of the classical photoreceptors (Semo et al., 2003), whereas in our experimental model melanopsin levels are greatly reduced (Sakamoto et al., 2004a). Such a result indicates that few remain photoreceptors (likely the green cones; Cicerone et al., 1979; Pinilla et al., 2005) and the *ipRGCs* with low melanopsin levels are still able to mediate circadian entrainment. Further studies are required to determine which photoreceptor type(s) is responsible for the entrainment and phase-shift of the locomotor activity rhythm.

The observation that the τ of the free-running period of *rdy* is shorter (about 1-h, Figure 2) than in *rdy*⁺ represents also an intriguing aspect of our work. A few authors have proposed that the changes in free-running period observed after photoreceptor degeneration or bilateral enucleation are due to the alteration in the relationships between the retinal and the SCN clocks (Lupi et al., 1999; Yamazaki et al., 2002; Lee et al., 2003). However, it is important to mention that previous studies have clearly shown that in *rdy* the retinal circadian clock is still functioning (Doyle et al., 2002; Sakamoto et al., 2004b). Such an observation suggests that it is unlikely that in *rdy* rats the changes that we have observed in the free-running period are a consequence of the presence or absence of the retinal circadian clock.

Another explanation for the observed changes in the free-running period observed in the RCS/N-*rdy* is that *Mertk* is somehow involved in the molecular mechanism that generates circadian rhythms. Indeed, our data show that *Mertk* transcripts are present in the SCN (Figure 3) and therefore it is possible to speculate that *Mertk* may interact with one or more clock genes. However a previous study has shown that the free-running period of the retinal circadian clock in *rdy* (24.1-h) is not different ($P > 0.1$) from that observed in the locomotor activity rhythm of *rdy*⁺ (Sakamoto et al., 2004b and this study). Therefore, we believe that it is unlikely that mutation in the *Mertk* gene may affect the period of oscillation in one circadian pacemaker but not in another one.

Finally, it is plausible to hypothesize that the changes in the free-running periods observed in *rdy* rats are the result of a secondary effect of photoreceptor degeneration on the developing SCN. Some support for this hypothesis can be found in our results which show that a shortening in the free-running period (similar to what observed in *rdy*) can be only obtained by bilateral enucleation of *rdy*⁺ at a specific post natal age (PN 21, see Figure 4) whereas enucleation of *rdy* at PN 21 or PN 40 did not induced any changes in the free running period (Figure 4).

As we have already mentioned, Lupi et al. (1999) reported that several circadian parameters are affected by the loss of the photoreceptors in *rdta* mice with respect to the wild-type: The free running period is significantly shorter (about 20 minutes), the magnitude of light-induced phase shift is significantly increased and, finally, the irradiance required to produce a saturating phase shift is also significantly increased. Our results well agree with those reported Lupi et al. (1999) in the *rdta* mouse, since the free-running period of the rat with dystrophic retina was significantly shorter and the magnitude of the light-induced phase shifts tended to be larger in *rdy* than in *rdy*⁺. Hence, we believe that the change in the free-running period observed in our experimental model is the result of inappropriate signals transmitted from the retina to the SCN, either *via* the retinal hypothalamic tract or *via* accessory pathways, such as the intergeniculate leaflet. However, it is important to mention that in *rd/rd* mice and in mice lacking of all the photopigments (i.e., rhodopsin, cone opsin and melanopsin) the free-running period is not different from the wild-type (Foster et al., 1991; Panda et al., 2003). These data clearly indicate that the effects of photoreceptor degeneration on the free-running period may be difficult to predict.

The bulk of our knowledge about circadian photoreception came from studies on mice in which one or more photoreceptors have been disabled with different genetic manipulations (recently reviewed by Peirson and Foster, 2006). These studies have provided important information about the functioning of this non-image forming visual system and they have led to the identification of a new class of retinal photoreceptors (i.e. *ipRGCs*). Nevertheless new experimental data suggest that the functioning and regulation of this new photoreceptive system is rather complex and may differ between species (Belenky et al., 2003; Bellingham et al., 2006; Chaurassia et al., 2005; Dacey et al., 2005). Therefore, we believe that the use of multiple animal models is needed to fully understand this new non-image forming visual system.

4. Experimental Procedure

4.1. Animals

Tan-hooded RCS/N-*rdy*⁺ (*rdy*⁺) rats homozygous for the normal *rdy* allele and congenic age-matched RCS/N-*rdy* (*rdy*) homozygotes with retinal dystrophy were used in this study.

Animals were raised from birth at Morehouse School of Medicine in a 12 h light –12 h dark (LD) cycle of illumination with light ($1 \times 10^1 \mu\text{W}/\text{cm}^2$) on from ZT 0 – 12. Food and water were available *ad libitum*.

4.2. Monitoring of running wheel activity

Animals were housed in cages equipped with a running wheel and a magnetic sensor system to detect wheel rotation (Mini-Mitter Co, Bend, OR). Wheel-running data were recorded in 5 min bins saved on a PC using specific software (*Tau*, Mini-Mitters Inc.) and were analyzed with *VitalView* package (Mini-Mitters Inc.) to determine rhythmicity and the free-running period (τ). The period was estimated by Chi-square periodogram analysis. For the experiment in which the animals were subjected to LD cycles of different irradiance, the reduction of the light intensity was achieved by wrapping with aluminum foil parts of the fluorescence bulbs. In order to ensure that the irradiance level and the spectral composition of the light were similar for all the experimental subjects, these two parameters were measured and then adjusted to the levels of each cage using a radio-spectrophotometer (USB 2000, Ocean Optics, Dunedin, FL).

4.3. Assessment of phase-shift

The phase shift in the free-running period of locomotor activity rhythms elicited by a single light pulse was used as an assay for photosensitivity of the circadian visual system. Male *rdy* and *rdy*⁺ rats were approximately 40 days old when they were transferred from the colony room into individual running wheel cages and housed in a light-tight room. The 12L:12D cycle was continued for 5 days, and then the animals were exposed to constant darkness (DD). After 14 days in DD (i.e. at PN 60 $\pm$ 2) individual animals were removed from their cages under infrared light exposed to a 15 min heat-filtered light pulse of varying irradiance (i.e. 1×10^{-3} , 1×10^{-1} and $1 \times 10^1 \mu\text{W}/\text{cm}^2$). Light was provided by optic illuminator equipped with a SolarcTM lamp (M50E011, 50 watts) which has a spectral distribution similar to the solar light. To obtain light of similar irradiance, the beam produced by the optic illuminator passed throughout a filtering glass (Enlightened Technology Associated, Inc.) that equally diffused the light over a surface of 25×25 cm (i.e. the surface of the cage containing the rat subjected to the light pulse). The spectral composition and irradiance within this area was monitored by a radio-spectrophotometer (USB 2000, Ocean Optics, Dunedin, FL). The light pulse was administered at circadian time (CT) 15 (the maximum point of phase delays in rat [Honma et al., 1985]). In order to ensure that each animal was pulsed at the exact circadian time, the following procedure was adopted. For each animal, CT 15 was determined based upon each animal's locomotor activity rhythm. CT 12 was defined as the time at which an animal began its daily bout of wheel running activity. Running wheel records were plotted, and a line was drawn through activity onsets in order to calculate CT 12 on the day of light pulse exposure. For each animal, CT 15 was calculated relative to CT 12. At the conclusion of the light pulse, rats were returned to the running wheel cages under infrared light and the locomotor activity was monitored for an additional 14 days. The magnitude of light-induced phase shift was determined by eye fitting through the steady-state activity onsets before and after the light pulse. The magnitude of the phase-shift was calculated by measuring the difference between the predicted and observed onset of activity.

4.3. Brain collection

rdy⁺ rats were sacrificed by over-anesthesia with Halothane and the brain were removed from the animals and immediately frozen (in situ) or dissected (real time quantitative RT-PCR). When the sampling occurred during the night time, animals were killed and enucleated under dim red illumination (less than 3 lux; 15W Kodak safe lamp filter 1A).

4.4. In situ hybridization

Blocks of brain containing the SCN regions were obtained from animals at ZT 10, immediately frozen using dry ice, and kept at -80°C until sectioning. Brains were then sliced into 20 µm-thick sections. For hybridization, at least five sections from the medial part of the SCN were used for each animal. The template for transcription was a 575 bp cDNA fragment of rat *Merk* (GenBank NM 022943) subcloned into a pZLI vector. The correct orientation of the construct was verified by sequence analysis and restriction enzyme digestion. Antisense and sense cRNA probes were generated using Fluorescein-12-UTP (Perkin Elmer Life Science) by *in vitro* transcription. The templates for transcribing RNA probes were made by linearizing recombinant plasmids.

Sections were immersed in pre-hybridization buffer containing 50% formamide, 5 × Denhardt's solution and 5X SSC for 2-h at room temperature. Then, the sections were hybridized with 75 µl of hybridization buffer, covered with a coverslip and incubated overnight in a humidified chamber at 59.5°C. The best labeling was obtained using a probe concentration of 1:100. Slides were then washed in 5 × SSC/50% formamide at 68°C for 1-h, in 2 × SSC for 1-h at 68°C and then incubated in 20 mg/ml RNase A at 37°C for 30 min followed by 2 × SSC for 1-h and 0.2 × SSC for 30 min (2 times) at room temperature. Slides were mounted and then viewed with a Zeiss Axioskop microscope equipped with epifluorescence.

4.5. Real-time quantitative RT-PCR

The area containing the SCN (about 1 mm²) was quickly dissected under bright-light illumination using a dissecting microscope, placed in a 5 µl drop of TRIZOL reagent (Invitrogen, Carlsbad, CA) sitting on aluminum foil, and rapidly frozen on dry ice. Total RNA was isolated using TRIZOL reagent (Life Technologies, Grand Island, NY) following 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 manufacturer's protocol. Each set of samples was simultaneously processed for RNA extraction, DNase I treatment, cDNA synthesis and PCR reaction.

Real-time quantitative PCR was carried out using an iCycler (Bio-Rad, Hercules, CA, USA). Detailed conditions have been described previously (Sakamoto et al., 2004a and 2005). As primers we used: *Merk* (GenBank accession number NM022943), 155 bp product 5'-tgacctgtgcagcatcaaac-3' (nt 899-908) and reverse 5'-atctttccacgaaatgc-3' (nt 1025-1041); *glyceraldehyde-3-phosphate dehydrogenase (gapdh)*, Genbank accession number X02231, 142 bp product) forward 5'-agacagccgcattcttctgt-3' (nt 24-43) and reverse 5'-tgatggcaacaatgtccact-3' (nt 146-165). Forty cycles of amplification were carried out following 15 sec of denaturation at 95°C. Once the temperature reached 95°C, it was decreased to 60°C, maintained for 45 sec, and then was raised to 72°C for 30 sec. Fluorescence was measured at melting temperature after each cycle not at the end of 40 cycles of amplification to allow comparison of fluorescence intensities among samples while their increase is within linear regression. Melting temperatures were 81 and 86°C for *Merk* and *Gapdh*, respectively. Quantification of *Merk* and *Gapdh* cDNAs was accomplished by comparing the threshold cycles for amplification of the unknowns to those of four concentrations of *Merk* and *Gapdh* standards using the *iCYCLER* software. After 40 cycles of reactions, PCR products were run on an agarose gel to verify

that a single amplicon of appropriate size was amplified. *Mertk* mRNA levels were normalized using *Gapdh* mRNA levels. In each experimental set, maximum values were represented as 100 and each value was normalized relative to the maximum value.

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Figure 1.

Effect of a single light pulse (15 min) at CT15 (white circle) on the phase of the circadian rhythm of wheel running activity in *rdy* (A) and *rdy*⁺ (B) rats at PN 60. *rdy* (N = 8, black bars) and *rdy*⁺ (N = 10, white bars) at PN 60 were exposed to 15 min of light (arrows) at there different irradiances (C). The magnitude of the phase shifts was determined by eye-fitted line drawn using the activity onset as reference point. No difference in the mean magnitude of the phase-shift was observed between the two different genotypes (*t*-tests, *P* > 0.1). The error bars in C represent the SEM.

Figure 2.

Representative actograms of running wheel activity in *rdy* (A) and *rdy*⁺ (B) rats maintained in DD. Mean free-running period $\pm$ SEM (C) of *rdy* (N = 21) and *rdy*⁺ (N = 18) and frequency distribution (D) in *rdy* and *rdy*⁺ rats maintained in DD. *rdy* rats have a shorter free-running period (*t*-test, *P* < 0.001) and no overlap of free-running period between the genotypes was observed.

Figure 3.

In situ hybridization for *Mertk* in the SCN of *rdy*⁺. An hybridization signal is clearly present in with the antisense (A) but not with the sense (B) probes. *Mertk* mRNA in SCN (C) did not show any significant variation during the 24 hrs (N = 4 for each time point, ANOVA, P > 0.1). The error bars in C represent the SEM.

Figure 4.

Enucleation of *rdy*⁺ at PN 21 induced a significant change (*t*-test, $P < 0.001$) in the free-running period (A, C), whereas enucleation at PN 40 did not affect the free-running period (*t*-test, $P > 0.1$, B, C). Enucleation of *rdy* at PN 21 or PN 40 did not affect the free-running period (*t*-test, $P > 0.1$ in all cases, C). The error bars in C represent the SEM.