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# Diurnal variations in the electroretinographic c-wave and retinal melatonin content in rats with inherited retinal dystrophy

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**Abstract.** The inability of retinal pigment epithelium to phagocytose shed photoreceptor disks is a cause of retinal degeneration in the Royal College of Surgeons rat; retinal pigment epithelial phagocytosis and disk shedding are regulated by the diurnal rhythm of retinal melatonin level. The diurnal rhythms of the electroretinogram (particularly that of the retinal pigment epithelial potential, the electroretinographic c-wave) and retinal melatonin content were thus investigated in Royal College of Surgeons rats from postnatal day 17 to 24, the period preceding retinal degeneration. The amplitudes of both the b- and c-waves of the electroretinogram fell significantly during the peak time of rod disk shedding and rose after the time of expected light off in the control and dystrophic rats. While the b-wave rhythms did not differ between the two strains, diurnal changes in the c-wave were significantly less distinct in the dystrophic rats than in controls. This difference may reflect lack of phagocytosis in dystrophic rats. Furthermore, the ERG c-wave was significantly larger and prolonged, and the retinal melatonin content higher, in dystrophic rats of this age group than in controls. It appears that retinal melatonin metabolism may play an important role in the maintenance of retinal pigment epithelial and photoreceptor function.

## Introduction

Retinal photoreceptors undergo continuous renewal, with old disks being cleared by phagocytosis in the retinal pigment epithelium (RPE) [1, 2]. Photoreceptor renewal follows a distinct circadian rhythm. Rod outer segments are shed in a burst, with a peak between 1 and 3 hours after the onset of light, although shedding can continue for at least 3 days in constant darkness [3]. In the rat, each RPE cell was estimated to phagocytose as many as 25,000–30,000 disks every day [2]. Failure of this process may be a cause of retinal and RPE degeneration in Royal College of Surgeons (RCS) rats [4, 5], which are one of the best studied models of retinal degeneration. It has been shown that retinal sensitivity, measured by the electroretinographic (ERG) a- and b-waves, decreases during the peak of rod outer

segment shedding in both normal and dystrophic RCS rats [6, 7]. However, phagocytosis is a process involving dynamic interaction between RPE and photoreceptor outer segments. We therefore examined whether the ERG c-wave (the light-evoked trans–pigment epithelial potential that depends on the integrity of the RPE and the photoreceptor outer segments) followed the circadian rhythm of rod disk shedding in dystrophic RCS rats and in their heterozygous control litter mates.

Many recent studies indicate that melatonin, a hormone synthesized by both the pineal gland and the retina, participates in regulation of circadian rhythmicity of disk shedding and RPE phagocytosis [8]. The question here is, then, whether the dysfunction in the RCS rats is associated with some abnormality of metabolism of retinal melatonin. Retinal melatonin content in dystrophic and control rats was therefore determined.

## Materials and methods

A total of 19 black-eyed dystrophic RCS-$p^+$ rats and 19 control RCS-$rdy^+p^+$ [9], purchased from the Institute of Ophthalmology, London, were used in this study. They were divided into two experimental groups.

In the first group, 10 dystrophic and 10 control rats aged 17–24 days were used for studying diurnal rhythm of the ERG and retinal melatonin content. Of the 10 rats in each strain, 5 were used in the morning and 5 in the evening series. However one dystrophic rat in the evening series died prematurely. Thus, for the melatonin study, 5 control and 5 dystrophic rats were used in the morning and 5 control and 4 dystrophic rats in the evening series. The ERG data obtained from one control and one dystrophic rat in the morning group and one control rat in the evening group had to be excluded because of electrode contact problems that occurred during the recording. Only 4 control and 4 dystrophic rats in both morning and evening studies were thus successfully completed in the ERG rhythm experiments.

In the second group, 9 control and 9 dystrophic rats of three different age groups were used for a developmental study of the ERG, as described below.

All of the rats used were raised in cyclic light (two overhead fluorescent lights, 300–400 lux at source) with a light = dark rhythm of 12 hours (lights on at 8 AM and off at 8 PM). Rats were transported in an illuminated box to our laboratory between 3 and 5 PM on the day before the ERG test. Lights were then turned off at 8 PM (the expected light-off time) and were kept off until the recording of the ERG was commenced on the following day.

### *ERG recording*

All experiments were carried out under dim red light (25W bulb with a

Kodak 1A red filter). Rats were anaesthetized with urethane, 1.5 g/kg given intraperitoneally. Urethane was chosen because it least affects the ERG c-wave [10]. Pupils were dilated with 1% (w/v) atropine. The use of phenylephrine was abandoned because it caused a gradual increase in the c-wave amplitude, as also found by Ruedemann and Noell [11]. Canthotomy was performed, and the eyelids were retracted by sutures of a specially constructed eyelid retractor. Each rat was placed in a stereotaxic frame with the head firmly held between ear bars and a mouth bar. Body temperature was monitored and maintained at approximately 37 °C by means of a thermostatically controlled heating pad. The electrocardiogram was also monitored throughout recordings and was used for judging the general physiologic condition of the animal.

All ERG recordings were made on only one eye of each animal while the other eye was occluded. The details of the ERG recording method are described elsewhere [12]. Briefly, a small contact lens connected to a silver–silver chloride half cell via saline-filled polystyrene tubing was used as an active electrode. A chlorided silver needle placed under the skin behind the lateral canthus of the other eye served as a reference.

The ERGs were evoked with light pulses produced by a cluster of 9 'ultra-bright' green light-emitting diodes (LEDs). The light-emitting diodes were composed together at a convergent angle, providing a uniform spot of light (the peak transmission at 565 nm) with a diameter of 5 mm on the rat cornea at a distance of 5 mm. The stimulus subtended a visual angle of 30°–40°. Mean luminance, calculated with variables of the rat eye determined by Dodt and Echte [13], was 2000 cd/m$^2$ (approximately 1.5–2 log units above the a- and c-wave threshold). Stimulus duration was 100 ms. The ERG responses were differentially amplified by a Medelec Sensor ER 94a with a frequency bandpass of 0.01–125 Hz. Time base was 10 seconds with 12.5% delay to assess the prestimulus baseline level.

*Study of diurnal rhythm of ERG*

After application of the electrodes, a period of approximately 30 minutes was allowed for stabilization of the ERG baseline before the experiment on diurnal rhythm began. Two recording periods, which included the expected dark-light and light-dark transition, were chosen for the study of the ERG diurnal rhythm: one in the morning and the other in the evening. The morning recording began at 7:30 AM, i.e., 0.5 hour before the expected light-on time, and ended at 10 AM, i.e., 2 hours after expected light-on time. The evening recording began at 7:30 PM, i.e., 0.5 hour before expected light-off time, and ended at 10 PM. The rats were thus dark adapted for 11 hours before the morning ERG recording and for 23 hours before the evening ERG recording.

*Developmental study of ERG*

In this separate experiment, ERGs were recorded at 5-minute intervals between 12 noon and 6 PM in fully dark-adapted eyes of 3 control and 3 dystrophic rats in each of the following three age groups: 17–18, 22–24 and 30–32 days old.

*Melatonin assay*

After the morning and evening ERG experiments, the eyes that had not been used for the ERG recording as well as those used for the ERG recording were enucleated, and the retinas were isolated from the RPE under dim red light for one melatonin assay. The time of isolation of the retinas thus corresponded to 2–4 hours after the time of expected light-on (10 AM to noon) or to 2–4 hours after the expected light-off time (10 PM to midnight). Of the 20 retinas taken in the morning, 10 (5 control and 5 dystrophic) were thus dark adapted for 14–16 hours, and the other 5 control and 5 dystrophic retinas received periodic flash stimulation during the ERG study. Similarly, of the 18 (10 control and 8 dystrophic) retinas taken in the evening, 9 (5 control and 4 dystrophic) were dark adapted for 26–28 hours, and the other 5 control and 4 dystrophic retinas received ERG stimulating flashes. The procedure of mixing the retinas that received some light flashes with the fully dark-adapted retinas is not ideal, since melatonin synthesis and metabolism are influenced by light stimulation. However, as the retinas taken from the dystrophic rats and those from the control rats received exactly the same stimuli, the comparison of melatonin content between the two groups may be allowed. The retinas were put in 100 μL of 50 mM Tris hydrochloride buffer containing 3 mM magnesium chloride and 1 mM edetate disodium (pH, 8.0) and frozen immediately in liquid nitrogen. They were subsequently homogenized on ice and centrifuged at 14 000 g for 5 minutes at 4 °C.

Supernatants were processed according to the protocol supplied by Stockgrand Ltd, University of Surrey, UK, with the method described by Fraser and associates [14]. Sheep antibody to melatonin was obtained from Dr J. Arendt, University of Surrey; 'cold' melatonin was obtained from Sigma Chemicals, UK; and tritiated melatonin was obtained from Amersham International plc, U.K. Protein assays were carried out according to the method of Miller [15]. Student's t-test was used as a statistical procedure.

## Results

*Diurnal rhythm of the ERG*

Figure 1A shows the relative changes in the amplitude of the dark-adapted

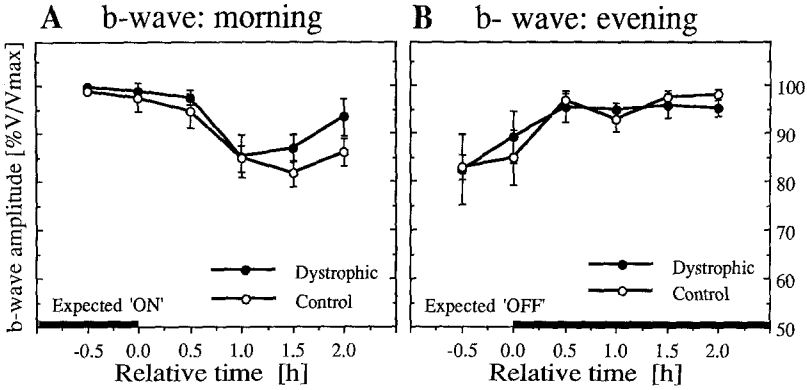


*Fig. 1.* Change in amplitude of the ERG b-wave (mean ± standard error of the mean) during the expected dark-light transition period in the morning (A) and during the expected light-dark transition period in the evening (B). Note that the b-waves of both dystrophic (n = 4) and control (n = 4) rats show a transient decrease 1–1.5 hours after the expected light-on time in A, whereas a gradual increase is seen during the expected dark-light transition in B. There was no significant difference between these diurnal changes in the b-waves of the control and dystrophic rats aged 17–24 days.

ERG b-wave during the dark to the expected light-on period in the morning, when the rod disk shedding activity is at its peak. The maximum amplitude of the b-wave obtained from each eye was designated 100% in these figures. The b-waves of both control and dystrophic rats fell between 1 and 1.5 hours after the expected light-on time; the reduction of the b-wave amplitude of control rats was 18.2% while that of dystrophic rats was 14.8%. This change was not significantly different between the control and the dystrophic rats. The changes in amplitude of the b-wave observed during the light to the expected light-off period are shown in Fig. 1B. The ERG b-wave of both control and dystrophic rats increased by 13% to 14% between 0.5 and 1.0 hours after the expected light-off time.

The relative change in the c-wave amplitude was plotted against the time of recording. The c-wave amplitudes were measured from the baseline. As Fig. 2A shows, the ERG c-wave gradually declined, reaching the lowest level 1 hour after expected light-on time in both control and dystrophic rats. However, this decrease was significantly ($p < 0.05$) larger in the control rats (35.1%) than in dystrophic rats (21.0%). Similarly, although the c-wave of both control and dystrophic rats increased during the transition period from light to the expected light-off time in the evening, the increase was significantly larger ($p < 0.05$) in control (20.2%) than in dystrophic (5.6%) rats (Fig. 2B).

A further important observation in the study of ERG development was that the ERG c-wave (recorded between noon and 6 PM) in the dystrophic rats from postnatal day 17–24 was significantly larger ($p < 0.05$) and more

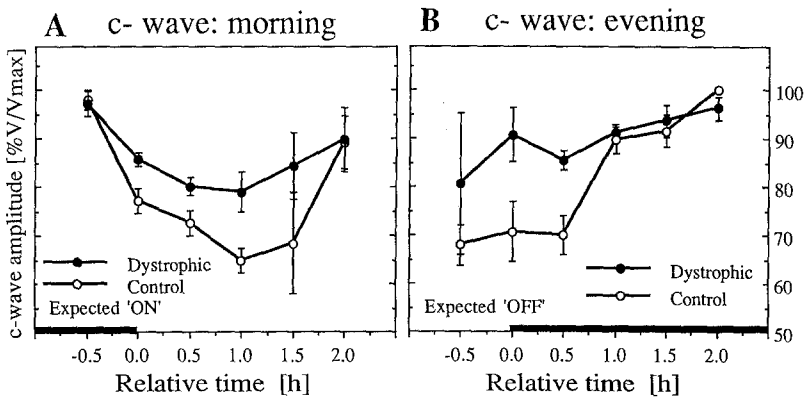


*Fig. 2.* Changes in the amplitude of the ERG c-wave (mean ± standard error of the mean) during the expected dark-light transition period in the morning (A) and during the expected light-dark transition period in the evening (B). Note that the c-waves of both dystrophic (n = 4) and control (n = 4) rats aged 17–24 days show a transient decrease 1–1.5 hours after the expected light-on time in A and a gradual increase between 0.5 and 1.0 hour after the expected light-off time in B. The diurnal change of the c-wave was significantly greater in the control rats than in the dystrophic rats for both transition times ($p < 0.05$).

prolonged ($p < 0.001$) than in the control rats. The mean amplitude of the c-wave of the dystrophic rats was 720.7 ± 43.3 μV (n = 6), while that of the control rats was 497.3 ± 69.2 μV (n = 6). In both control and dystrophic groups, 3 rats were taken from postnatal day 17–18 age group and 3 from the day 22–24 group. Mean implicit time of the c-wave was 3.2 ± 0.1 seconds in the dystrophic rats and 2.1 ± 0.2 seconds in the control rats. All components of the ERGs in the dystrophic rats thereafter rapidly declined with age, and by day 35 they were much smaller than those in the control rats (Fig. 3).

*Melatonin content in the retina*

Figures 4A and 4B show the melatonin content in 10 retinas extracted from 5 control rats and 10 from 5 dystrophic rats between 10 AM and noon and 10 control and 8 dystrophic retinas isolated between 10 PM and midnight. Melatonin content, both in the morning and in the evening, was significantly higher ($p < 0.05$) in the dystrophic retinas (morning mean ± standard error of the mean], 74.0 ± 11.5 pg/mg of protein; evening, 84.4 ± 18.5 pg/mg of protein) than in the control retinas (morning, 39.9 ± 4.0 pg/mg of protein; evening, 42.5 ± 9.8 pg/mg of protein). The mean melatonin content was slightly higher in the retinas taken in subjective dark (B) than in subjective light (A), although this difference was not significant.

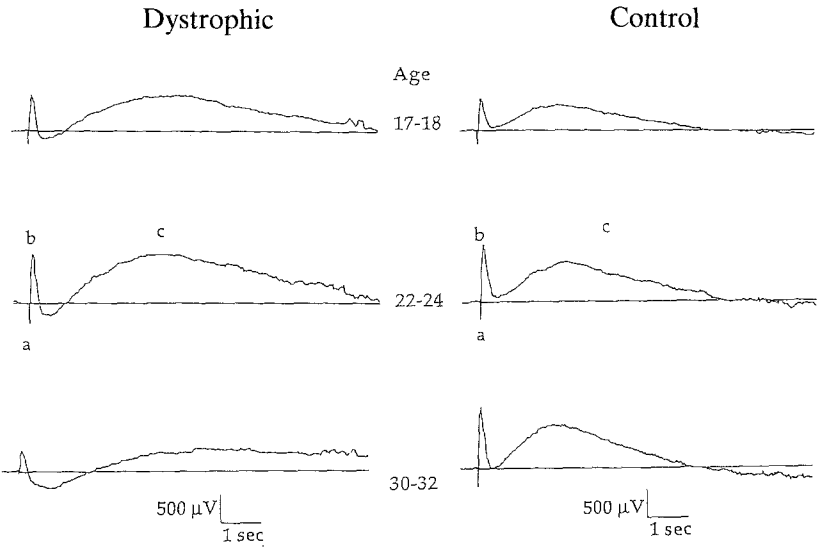


*Fig. 3.* Examples of ERGs obtained from dystrophic and control rats of three different age groups (in days). Each trace is an average of responses obtained from three rats. Note that the c-waves of the dystrophic rats aged 17–18 days and 22–24 days are significantly larger and more prolonged than those of their respective control, while the b-waves of the control and dystrophic rats show no difference. The ERG of dystrophic rats 30–32 days old is already considerably smaller than that of control rats of the same age. The horizontal line drawn across each ERG trace is the baseline from which the a- and c-wave amplitudes have been measured. The b-wave is measured from the trough of the a-wave.

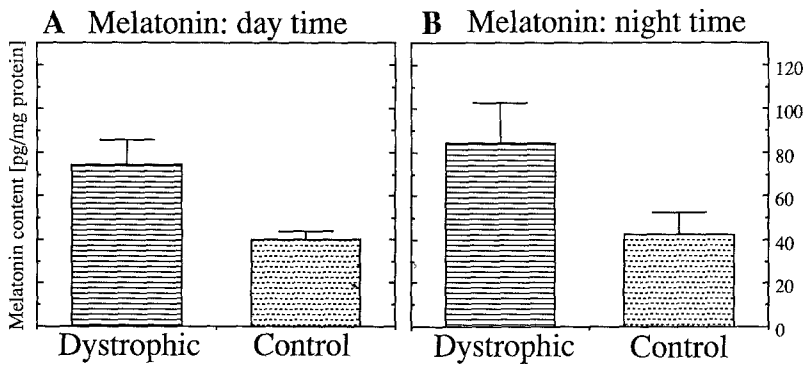


*Fig. 4.* Content of melatonin (mean ± standard error of the mean) determined in 10 control and 10 dystrophic rat retinas isolated during subjective daytime (10 AM to noon) (A) and in 10 control and 8 dystrophic retinas isolated during subjective nighttime (10 PM to midnight) (B). Note that the mean melatonin content of the dystrophic retina is significantly higher ($p < 0.05$) in both A and B.

## Discussion

We found that, while the ERG c-wave of the control rats showed a distinct change associated with the diurnal rhythm of rod disk shedding and RPE phagocytosis, that of the dystrophic rats did not (Figs. 2A and 2B). In contrast, confirming the finding of Sandberg and associates [7], no difference in the diurnal rhythm of the ERG b-wave was found between the dystrophic and the control rats (Figs. 1A and 1B). These results are consistent with the abnormality in the RCS rats being located in the RPE rather than in the photoreceptors [5], as was proposed by Sandberg and associates [7]. These authors believed that diurnal changes in the a- and b-waves of the ERG reflected the photoreceptor disk shedding process, which is not affected in early stages of degeneration in the RCS rats [16].

It is not yet known whether the diurnal rhythm of the ERG c-wave is associated with the rhythm of phagocytosis of shed disks by the RPE. However, the ERG c-wave represents light-evoked change in the algebraic sum of the trans–pigment epithelium (positive) and the transretinal (negative) potentials [17]. One might then speculate that, in the normal retina, the c-wave would be expected to change diurnally if changes in resistance of the apical and basal membranes of the RPE occurred and were associated with ingestion of shed disks. A reduction in the phagocytosis could, therefore, cause less diurnal variation in the ERG c-wave. A study of the diurnal rhythm of the RPE membrane resistances in association with the RPE phagocytosis would be of interest in this context.

Our finding that the ERG c-wave, as a whole, is abnormally enhanced and prolonged in the dystrophic rats (Fig. 3) is, furthermore, in agreement with the early work of Arden and Ikeda [18]. They found that the light-evoked changes in the corneofundal potential in RCS rats from postnatal day 18 to 24 were abnormally increased. The light-induced rise in the corneofundal potential then declined rapidly with age, in association with the progressive degeneration of the RPE and photoreceptors. These authors suggested that the supernormal light rise in the corneofundal potential in the young dystrophic rats was an electrophysiologic correlate to the increased extracellular accumulation of rhodopsin-containing lamellae [4]. This explanation is also applicable to our finding of the abnormally enhanced c-wave in the same animals at the same age. However, the present results on the diurnal variation of the c-wave and its association with the circadian rhythm of disk shedding and RPE phagocytosis add an extra dimension. They provide a means of relating these phenomena to the lack of phagocytic mechanisms in the RPE of the dystrophic rats.

The cause of the failure to phagocytose shed disks by the dystrophic rat RPE is not known. Our study showed, however, that retinal melatonin levels are abnormally raised in these rats. As melatonin has been shown to inhibit phagocytosis by the RPE in culture [19], the abnormally increased

melatonin level might provide increased inhibition of phagocytic activity. However, whether inhibition of RPE phagocytosis by melatonin is a direct action of melatonin or the action of a substance associated with melatonin is debatable. For example, as melatonin is synthesized in the photoreceptors in a process requiring cyclic adenosine monophosphate [20], changes in cyclic adenosine monophosphate metabolism rather than melatonin might be responsible for the abnormality [21]. Indeed, the levels of cyclic nucleotides have been shown to be elevated in the dystrophic rat retina during the early stages of degeneration [22]. The increase in melatonin content in the RCS rats might also be due to a defect in the enzymatic degradation of melatonin within the eye [23].

Finally, as the levels of melatonin and dopamine in the retina are inversely related [24, 25], abnormalities in regulation of the retinal melatonin level could be due to a deficiency of retinal dopamine, another putative modulator of the photoreceptor renewal process. Indeed, the increased level of melatonin that we found in the dystrophic retina may be a consequence of reduced dopamine release in the retina that is programmed to degenerate. Thus, our findings lend some support to the early observations that melatonin promotes light-induced retinal degeneration, while the dopamine agonist bromocriptine increased survival of the RCS rat retinas [26]. However, further studies are necessary to shed light on the interesting relationship between the retinal dopaminergic system and retinal melatonin and degeneration.

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