Characterization of the Day-Night Variation of Retinal Melatonin Content in the Chick

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By monitoring two time points (one at mid-light and the other at mid-dark), the day-night variation of melatonin content in the retina of 19-day-old chicks was characterized. Melatonin was detected in the retina plus attached pigment epithelium by a specific radioimmunoassay, and its identity was verified by high performance liquid chromatography with electrochemical detection. Melatonin content in the posterior pole of the eye showed a fivefold day-night variation, with high levels during the dark period of diurnal lighting. Light exposure during the dark period lowered the normally high nighttime value; maintenance of darkness during the normal light period did not alter the low melatonin values typical of mid-light. Pineal melatonin content responded similarly to the above lighting manipulations. Neither pinealectomy nor optic nerve transection had an effect on retina-pigment epithelium melatonin or its light-dark rhythm. We next examined the relative contributions of retinal and pineal melatonin to blood levels. Pinealectomy reduced the normally high mid-dark plasma melatonin value by 80%. The addition of bilateral enucleation reduced the mid-dark value by another 9% of control values. The day-night variation of retina-pigment epithelium melatonin was first evident in the embryo 2 days prior to hatching and persisted through adulthood. It was concluded that the chick retina from the latter stages of embryonic development is capable of rhythmically synthesizing melatonin; that retinal melatonin content displays a photically controlled circadian rhythm in phase with, but independent of, the pineal gland; and that the retinal rhythm is not regulated by afferent optic nerve fibers. The pineal gland is the major source of plasma melatonin in the intact chick, with at most a small contribution from the retina. Invest Ophthalmol Vis Sci 24:294-300, 1983

It is well established that the major source of melatonin for homoiotherms is the pineal gland. This organ synthesizes melatonin from serotonin by use of two enzymes, N-acetyltransferase (NAT) and hydroxyindole-O-methyl transferase (HIOMT), with NAT controlling the well-described daily oscillations of pineal melatonin production in rats and chickens. The pineal melatonin rhythm, in turn, is reflected accurately in blood, urine, and cerebrospinal fluid, with high levels occurring at night. Interestingly, the retinae of a number of vertebrate species have been known for some time to contain the enzymatic machinery necessary for melatonin biosynthesis. A resurgence of interest in this retinal capability has led to the recent findings of large daily rhythms in NAT activity and melatonin content in the chick retina.

Our interests in melatonin physiology and the potential endocrine functions of the retina led us to characterize the retinal rhythm of melatonin content in the chick by examining its response to alterations in environmental lighting, by delineating the influence of the pineal gland and of retinal optic nerve afferents in the generation of the eye rhythm, and by studying the ontogeny (embryo through adult) of the day-night variation in retinal melatonin content. An important aim of our studies was to assess the relative contributions of retinal and pineal melatonin to circulating levels of the hormone.

Materials and Methods

Animals

Zero day fertile eggs and 12-day-old White Leghorn Chicks (Gallus domesticus) were purchased from Spaffs, Inc. (Norwich, CT). Eggs were kept in a warmed (37 C), humidified incubator equipped with an automated lighting system; intensity of illumination at the mid-portion of the incubator was 600 lux. Chicks were communally housed in a temperature-controlled brooder that was quartered in a light-controlled room; intensity of illumination was 400 to 600 lux at the food and water bins. Blind chicks (see
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Fig. 1. Photic regulation of pineal, ocular, and plasma melatonin.
At 19 days of age animals were killed at mid-light or mid-dark. Animals were also killed at the subjective mid-dark time with lights left on throughout the experimental dark period or at the subjective mid-light time with lights left off throughout the experimental light period. Eye (middle panel) refers to the posterior hemisphere of one eye. Data are presented as mean ± SE of six to eight chicks in each group.

“Surgical Procedures”) were housed within a fenced off portion of the communal brooder in order to protect them from sighted animals. Purina Chick Starteen and water were available at all times. Unless otherwise specified, the daily light-dark cycle consisted of 12 hrs of light per day (LD 12:12) with lights on from 0600 hrs EST. For experiments involving hens, animals were reared by the animal husbandry department of the University of Connecticut at Storrs and housed there in the diurnal lighting cycle described above.

Surgical Procedures
Chicks were anesthetized with pentobarbital (40 mg/kg, IM), and the heads were secured in a rat ste-
Fig. 2. Effects of pinealectomy (Pnx) on the day-night variation of ocular melatonin. At 14 days of age, animals were divided into two groups: one group was pinealectomized and the other received sham operations. Five days later animals from each group were killed at mid-light or mid-dark. At each time point, eight pinealectomized animals and four sham animals were used. Data are presented as mean ± SE.

was validated by demonstrating (1) parallel inhibition curves between serial dilutions of extracted nighttime plasma samples and the melatonin standard, and (2) quantitative recovery of 125 pg, 250 pg, and 500 pg of melatonin from 1-ml samples of pooled daytime plasma. The extraction efficiency for all samples was greater than 95%; values reported are not corrected for the small loss during extraction. The limit of assay sensitivity was 0.5 pg/tube. The within and between assay coefficients of variation were 10 and 15%, respectively.

Chemical Validation of Retinal Melatonin by High-Performance Liquid Chromatography (HPLC)

Radioimmunoassay analysis of retina-pigment epithelium melatonin was corroborated by HPLC with electrochemical detection using the method of Mefford and Barchas11 with modifications (Fig. 3). Pooled chloroform extracts of ocular tissue were dried under vacuum in darkness and redissolved in 0.1 ml HCl. After centrifugation, aliquots of the supernatant were injected into a 0.4 × 30 cm micron Bondapak C18 reversed phase column (Waters Assoc., Milford, MA) eluted with a mobile phase consisting of 0.1 M sodium acetate buffer (pH 4.7), 0.1 mM EDTA, and 25% methanol at a flow rate of 1.5 ml/min. A LC-4 electrochemical detector (Bioanalytical Systems, West Lafayette, IN) with a glassy carbon electrode was used at a potential of +0.90 V. Peaks were identified by retention time and melatonin quantified by peak height.

Experimental Protocols

Two time points (one at mid-light and the other at mid-dark) were used to monitor daily melatonin rhythms in the eye, pineal, and plasma. These time points correspond to the nadir and peak, respectively, of the daily melatonin rhythms in all three tissues in the chicken, as studied in diurnal lighting.2,8,14 The term ocular melatonin is used in the text to signify melatonin content in the posterior hemisphere of the eye. Other experimental details appear in the “Results” section and the figure legends.

Statistical Methods

Statistical analyses were performed using the Mann-Whitney U/Wilcoxon nonparametric test.

Results

Photic Regulation of Pineal, Ocular, and Plasma Melatonin

Day-night variations of melatonin concentrations in the pineal gland, eye, and plasma were examined in chicks exposed to diurnal lighting and killed at
either mid-light or mid-dark. In this lighting cycle, there are prominent, synchronous day-night oscillations in pineal, ocular, and plasma melatonin, with high values at night (Fig. 1); the magnitudes of the nocturnal excursions are 17-fold in the pineal, five-fold in the eye, and tenfold in plasma.

Light suppression of nocturnal melatonin production was examined in the chick by killing animals at the mid-dark time with lights left on throughout the experimental dark period. This manipulation suppresses plasma and ocular melatonin levels such that they become similar to normal mid-light values (Fig. 1). The treatment also lowers pineal melatonin levels, but not to normal midday values.

To determine whether melatonin levels can decrease without a light cue, animals were killed at the mid-light time with lights left off throughout the experimental light period. This results in low ocular and plasma melatonin levels like those normally found during the light portion of the day (Fig. 1). Pineal melatonin content is also reduced, compared to mid-dark levels, in this environment, but the resulting value is higher than that usually found at mid-light.

**Effects of Pinealectomy on Ocular and Plasma Melatonin Levels**

Pinealectomy does not alter the day-night variation in ocular melatonin content (Fig. 2). In contrast, pineal removal has a marked effect on the normally high mid-dark plasma melatonin concentration, lowering it by 80% (Fig. 4). Pinealectomy does not, however, alter the normally low mid-light plasma melatonin level. Interestingly, a small but significant \( P < 0.01 \) day-night variation in plasma melatonin levels, with the high value at night, is still evident in pinealectomized chicks.

**Effects of Pinealectomy plus Blinding on Plasma Melatonin**

To examine the contribution of the retina to circulating melatonin levels, blind-pinealectomized animals were killed at either mid-light or mid-dark, and their circulating melatonin concentrations compared to those of pinealectomized animals. Beyond the reduction incurred by pinealectomy alone (see preceding section), blinding causes a further 9% reduction of the mid-dark plasma melatonin value (Fig. 4). This procedure does not alter the mean mid-light plasma melatonin concentration. Blind-pinealectomized chicks do not appear to exhibit a day-night variation in plasma melatonin levels.

**Effects of Optic Nerve Transection on Retina-Pigment Epithelium Melatonin**

The role of optic nerve afferents to the retina in the generation of the day-night variation in retina-pigment epithelium was also examined. As shown in Figure 5, optic nerve transection does not affect the eye rhythm, as studied under diurnal lighting conditions.

**Ontogeny of the Day-Night Variation in Retina-Pigment Epithelium Melatonin**

Developmentally, a significant \( P < 0.01 \) day-night variation in retina-pigment epithelium melatonin content, with high nighttime levels, is first detected in the late embryo, 2 days prior to hatching (Fig. 6). The increase in the magnitude of the variation during maturation results primarily from an increase in the mid-dark value. A similar developmental pattern was found for pineal melatonin content (data not shown).
Fig. 5. Effects of optic nerve transection on the day-night variation of retina-pigment epithelium melatonin. Optic nerve transection (right eye) and sham operation (left eye) were performed in each animal at 14 days of age. Five days later animals were killed at mid-light or mid-dark. Data are the mean ± SE of six to eight animals in each group.

Examination of retina-pigment epithelium melatonin content in hens showed that the day-night variation is prominently manifested in the adult animal.

Discussion

After Binkley et al.4 discovered a prominent daily rhythm in NAT activity in the chick retina, Hamm and Menaker8 showed that both NAT activity and melatonin content exhibit synchronous daily retinal rhythms in this species and respond similarly to manipulations of the daily light-dark cycle. They also showed that the retinal NAT rhythm persists after pinealectomy. The results presented here confirm and extend those observations.

By monitoring melatonin concentrations at two time points in the posterior pole of the eye and in the pineal gland of each animal, we have shown that the day-night variations in both organs are synchronous and regulated by environmental lighting in a similar manner; high nighttime levels are suppressed by light whereas the rhythms persist in darkness. We also dissected retina from pigment epithelium and found, as others have,15,8 that in the chick ocular melatonin and HIOMT are localized to the retina (Sagar and Reppert, unpublished data). The results of our pinealectomy experiment show that the pineal gland is not the source of retinal melatonin since ocular melatonin levels continue to oscillate in animals lacking pineals. Taken together, the findings to date indicate that the chick retina rhythmically synthesizes melatonin and that retinal melatonin content displays a photically controlled circadian rhythm in phase with, but independent of, the pineal rhythm.

The results of our developmental study suggest that melatonin is produced rhythmically by the retina as early as the latter stages of embryonic development. This coincides with the time that day-night variations...
in pineal NAT activity and in pineal melatonin content are first detectable in the chick embryo. Thus, it seems that the rhythmic production of melatonin by the retina and pineal gland exhibit similar developmental patterns.

An interesting question arises as to the site of the endogenous oscillator that generates the retinal rhythm. It does not reside in the pineal gland since the retinal rhythm persists after pinealectomy. Another possibility is that the rhythm might be generated by neural input from the brain. One way that neural information could reach the retina is via optic nerve afferents, which are prevalent in avian species. This does not appear to be the case, however, since optic nerve transection does not affect the eye rhythm in diurnal lighting. Other possible neural inputs to the retina that could be considered in this regard, such as sympathetic fibers, require further evaluation. It is worth noting that Binkley and coworkers have shown by using patch experiments and monitoring retinal NAT activity that within an individual chick the two retinas can respond to light independently of each other, suggesting that each eye contains an endogenous mechanism for rhythm generation.

A major focus of our study was to examine the contribution of retinal melatonin to circulating concentrations of the substance. Such a contribution seemed possible since melatonin readily passes through cell membranes and many biologic barriers, including the blood-brain barrier and the placenta; this diffusibility is due to the nonpolar, lipophilic nature of this small molecule (mol. wt. 232). Also, based on studies with the rat pineal gland, melatonin is not stored in secretory vesicles, but instead diffuses rapidly into blood upon production.

We examined the contribution of retinal melatonin to blood levels by first showing that our RIA system detects a large day-night variation in plasma melatonin concentrations in the intact chick (Fig. 1). The variation that we found (mean mid-day value of 31 pg/ml; mean mid-dark value of 322 pg/ml) is in good agreement with those reported by three different laboratories (range of mid-light values 10 to 50 pg/ml; range of mid-dark values 210 to 350 pg/ml) where bioassay and two other melatonin RIA systems were used. We next showed that the pineal gland is the major source of circulating melatonin concentrations at night, since the increase at mid-dark is nearly abolished in pinealectomized animals; this again is consistent with an earlier report. We also found that retinal melatonin contributes at best a small, albeit statistically significant, amount of melatonin to the mid-dark circulating levels. Furthermore, animals lacking pineals exhibit a detectable day-night variation in blood melatonin, with the higher levels at night. Whether this small variation actually represents a daily rhythm of retinal origin or is of physiologic significance is not possible to determine from the two time points examined in our experiment.

Based on the biophysical properties of melatonin discussed above, the apparently small retinal contribution to circulating levels of the hormone was unexpected. If one estimates from Figure 1 the amount of melatonin produced by each eye, assuming that melatonin levels reflect production rates in the retina, as suggested by the parallel rises and falls of NAT activity and melatonin content in the retina, then we would have expected both retinæ together to be responsible for roughly 50% of the high nighttime circulating melatonin concentrations. That this is not the case is difficult to explain. A possible explanation is that retinal melatonin might be metabolized locally within the eye.

Since our results suggest that the contribution of retinal melatonin to blood is quite small, the most likely function of retinally derived melatonin is in the regulation of rhythmic phenomena within the eye itself. There are three processes in the retina and pigment epithelium of various species known to display circadian rhythms: pigment migration, the elongation and contraction of the inner segment of photoreceptors (retinomotor movements), and rod outer segment disk shedding. There is some evidence in nonavian species that exogenously administered melatonin can influence each of these processes. Pigment migration in mammals and retinomotor movements in amphibians can be influenced by melatonin. Moreover, rod outer segment shedding in rats can be modulated by systemically administered melatonin. The role of retinal melatonin in the regulation of these mechanisms in the chick deserves systematic study. Furthermore, the hypothesis that in other species including mammals, the retina may be a target organ for melatonin should be tested.

Key words: retina, pineal gland, melatonin, circadian rhythms, chicks

References


