Circadian regulation of chick electroretinogram: effects of pinealectomy and exogenous melatonin

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McGoogan, Jennifer M., and Vincent M. Cassone. Circadian regulation of chick electroretinogram: effects of pinealectomy and exogenous melatonin. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R1418–R1427, 1999.—Melatonin is an important component of the avian circadian system. This study investigates the effects of pinealectomy (Pin-X) and melatonin implantation (Mel) on electroretinogram (ERG) rhythms in chicks. Feeding rhythms were monitored to obtain a phase reference for ERG recordings. Pin-X and Mel had little or no effect on feeding rhythms. Sham-operated Pin-X and vehicle implantation had no effect on ERG rhythms in the light-dark (LD) cycle or constant darkness (DD). ERG a- and b-wave amplitudes were higher during the day than during the night. The a- and b-wave implicit times were shorter during the day than during the night. a-Wave sensitivity was higher during the night than during the day, whereas b-wave sensitivity was not rhythmic. Pin-X abolished the circadian rhythm of b-wave amplitude and implicit time in DD but had no effect on a-wave rhythmicity. Mel abolished the rhythm of b-wave amplitude and of a- and b-wave implicit time in DD. Neither treatment affected ERG in LD. These results suggest that the circadian system regulates rhythmic visual function in the retina at least partially through Mel. The role played by the pineal gland and Mel may be specific to some physiological modalities (e.g., vision) while not influencing others (e.g., feeding).

ACCURATE DAILY TIMING of behavioral, physiological, and metabolic events is critical for the survival of all organisms. Timekeeping is accomplished by perception of environmental time cues (usually light) that daily reset endogenous pacemakers, a process called entrainment. These entrained oscillators, in turn, drive output rhythms with an appropriate phase angle to the light-dark (LD) cycle (26). In birds, putative photoreceptors responsible for entrainment reside in the ocular retinas, pineal gland, and specialized photoreceptive organs in the tuberal and sepal regions of the brain. Circadian oscillators have also been identified within the retinas, pineal gland, and brain, most probably in the hypothalamic visual suprachiasmatic nuclei (vSCN; see Refs. 6, 9, and 11). The relative importance of each structure and the strength of coupling between them vary among avian species; however, the neural and neuroendocrine interactions among these components are crucial for circadian system stability (6, 11). For example, pinealectomy (Pin-X) completely abolishes circadian patterns of activity in oscine passerine birds, disrupts or alters the circadian period in columbiform birds, and has little or no effect on the locomotor rhythms in galliform birds (6, 12, 14, 15).

Although a multitude of biological rhythms has been characterized in various avian species, the mechanism by which the circadian system drives rhythmic phenomena is completely unknown. One of the major outputs or components of circadian systems is the indoleamine hormone melatonin. Interestingly, melatonin is rhythmically synthesized in and released from the photoreceptive cells of the retina and pineal gland, likely the circadian oscillators themselves, almost exclusively during the night (1, 4, 16, 18). Thus melatonin is produced by the photoreceptive oscillators within the circadian system and is also its major output (18).

The major sites of melatonin action in birds are structures involved in visual processing. Melatonin binding studies using the agonist 2-[125I]iodomelatonin have shown that high-affinity binding occurs in visual system structures in the brain of several avian species (7, 8, 29, 30). Three putative melatonin receptors have been isolated, cloned, and characterized in chicks, Gallus domesticus (28, 29). In situ hybridization and Northern blot analysis reveal that the retina and visual system structures of the brain are the primary sites of central nervous system melatonin receptor expression (24, 28, 29). These structures are themselves rhythmic in 2-deoxy-[14C]glucose (2-DG) uptake such that uptake is greater during the day than during the night (19). Furthermore, melatonin injections given during the day can suppress 2-DG uptake to nighttime levels (7), and daily melatonin administration synchronizes rhythms of visual system 2-DG uptake in Pin-X sparrows, Passer domesticus (20).

These data strongly suggest that visual structures are regulated by the circadian system through the actions of melatonin, but they do not address whether the visual system responses to light are regulated similarly. The electroretinogram (ERG) is a field potential recorded from the surface of the eye that reflects the electrical response of the retina to a light stimulus (13, 17). It is an easily reproducible and scientifically informative measure of visual processing at the level of the retina. The initial a-wave of the ERG is a downward deflection that reflects the electrical response of the photoreceptor layer to the light stimulus. The subsequent b-wave is an upward deflection that reflects the response of the rest of the retina (13). Although the inner neural retina contributes to this b-wave response, the majority of the current is carried by glial Müller cell K⁺ uptake from the extracellular space (23, 27).
Chick ERG is rhythmic on a daily and circadian basis; a- and b-wave amplitudes are higher, implicit times are shorter, and a-wave sensitivity is lower during the day than during the night (21, 31, and J. J. McGoogan, W. Q. Wu, and V. M. Cassone, unpublished observations). In pigeons, Columba livia, ERG b-wave amplitude is similarly rhythmic such that the visual response has a higher amplitude during the day than during the night (3). Taken together, these results strongly suggest that the circadian system regulates avian visual processing.

To directly test the hypothesis that the clock regulates visual function at the level of the retina via melatonin, two experiments were performed. First, ERGs were recorded from sham-operated (Sham) and Pin-X chicks maintained in a 12:12-h LD cycle and in constant darkness (DD) to determine whether the pineal gland regulates the rhythms in retinal function. Second, ERGs were recorded from birds implanted with vehicle (Veh) or with crystalline melatonin (Mel), which provides a pharmacologically high level of blood melatonin, in LD and DD to determine whether melatonin regulates retinal function.

METHODS

Animals and activity recording. Male White Leghorn chickens (n = 36) were obtained on the first day posthatch (Hy-Line International, Bryan, TX) and were maintained in heated brooders on a 12:12-h LD cycle until 3 wk old (lights on at 6:00 AM and lights off at 6:00 PM Central Standard Time) with food and water constantly available. Chicks were then moved to another room where they were housed individually in cages equipped with feeders modified for continuously recording feeding activity. Feeding activity data were collected in 10-min bins by computer with the Datquest III hardware and software package (Mini-Mitter, Sunriver, OR) and were analyzed with Tau software (Mini-Mitter). The free-running period was calculated by χ² periodogram analysis. Feeding activity was used as a phase reference marker for taking ERG recordings. The middle of the active phase was designated zeitgeber time (ZT) 6 in LD and circadian time (CT) 6 in DD. The middle of the inactive phase was designated ZT18 in LD and CT18 in DD. For all surgeries, blood sampling, and ERG recordings, chicks were anesthetized with xylazine (Sigma Chemical, St. Louis, MO) given as an intramuscular injection at a dose of 100 mg/kg body wt.

Pin-X surgeries. Birds (n = 12) were anesthetized and placed in a stereotaxic instrument to prevent movement during surgery. A single incision was made in the scalp mid sagitally behind the comb, and the skull was exposed and dried. The pineal gland was exposed by craniotomy and was sealed with Silastic gel (Dow Corning), and dried. The capsules were kept dry at −20°C until the day before implantation when they were moved to physiological saline at 4°C. Intact chicks (n = 24) were anesthetized as above, and an incision was made in the skin on the back of the neck. Capsules were rinsed in 70% ethanol, and two capsules were inserted subcutaneously. Veh birds (n = 12) received empty capsules, whereas Mel birds (n = 12) received capsules containing Mel. The incision was closed and treated with topical antibiotic (Neosporin). Chicks were allowed to recover for 1 wk before ERG recordings commenced. After all ERG recordings were completed, blood samples were taken at midday and midnight from the brachial vein of each implanted bird. Serum was collected and frozen at −20°C until analyzed for melatonin content. Serum melatonin content was measured by RIA (C1Dtech Research, Mississauga, Ontario, Canada), which had been validated for chick serum previously (5).

ERG Recordings. ERGs were recorded from all chicks six times per day, or every 4 h. Each chick was recorded for only one time each day to prevent possible compounding effects of anesthesia. At each time point, a single chick was anesthetized and immobilized in a stereotaxic instrument (Stoelting, Wood Dale, IL). A 3-mm active gold cup electrode (Astro-Med, West Warwick, RI) was placed on the inferior corneal surface of the right eye, and reference and ground needle electrodes were placed in the comb and breast muscle, respectively. Light stimuli, 10 µs in duration at 10 different intensities, were delivered with a photostimulator (Astro-Med) placed 10 cm away from the surface of the eye. The intensity of the stimuli was controlled with a series of neutral-density filter combinations (Bogen Photo, Ramsey, N.J.). Light intensity was measured with a Biospherical Instruments (San Diego, CA) QSL-100 radiometer. Visually evoked signals were amplified and digitized using MacLab hardware and software (MacLab, Milford, MA) on a Power Macintosh computer. Five ERGs were recorded and averaged for each bird at each time point at each intensity.

Statistical analysis. Periods of feeding activity of animals in the four treatment groups were compared with the Student's t-test, and the P < 0.05 level was used to determine significance.

In all cases, a-wave amplitude was measured as the absolute value of the voltage relative to zero, and b-wave amplitude was measured as the absolute value of the voltage difference between the a- and b-wave peaks. The a- and b-wave implicit times were measured as the time from the onset of the stimulus to the peak voltage. The response amplitudes and response implicit times of both the a- and b-waves were averaged over all birds at each time. The average response amplitudes at each time and the average response implicit times at each time were plotted against the log stimulus intensity to create intensity response curves. Averages of the amplitudes at the higher-intensity stimuli were plotted against time of day to create amplitude curves. Averages of the implicit times at all stimulus intensities were plotted against time of day to create amplitude curves. To produce sensitivity curves, the value of the stimulus required to give the half-maximal response amplitude was calculated and called EI50 or median efficacious intensity. Sensitivity values were calculated by first adjusting for the change in amplitude over time of day by calculating the percent maximal response for all intensity response amplitude data. Multiple regression analysis was then run on these percentage data for both a- and b-waves of each individual bird at each time. A second-order regression model was used for both a- and b-wave percent response amplitude data. From the
regression equation calculated for each wave for each bird at each time of day. EI \textsubscript{50} values were derived. For sensitivity curves, EI \textsubscript{50} was plotted against time of day. Note that lower EI \textsubscript{50} values reflect higher sensitivity.

All statistical analysis was performed on SAS statistical analysis software (SAS Institute, Cary, NC). All points represent averages of the response of \(n = 5-6\) animals \(\pm SE\). Amplitudes, implicit times, and sensitivities were compared over time of day and between experimental groups using the nonparametric Kruskal-Wallis and nonparametric post hoc Mann-Whitney \(U\)-tests. In all cases, the \(P < 0.05\) level was used to determine statistical significance.

RESULTS

Feeding activity. Figure 1 depicts the feeding activity of representative Sham (Fig. 1A), Veh (Fig. 1B), Pin-X (Fig. 1C), and Mel (Fig. 1D) chicks in LD and in DD. Feeding activity of all chicks was rhythmic and entrained in LD, with birds feeding almost exclusively during the day. In DD, all chicks retained rhythmicity, and their feeding rhythms ran free with a short period: Sham 23.3 \(\pm\) 0.1 h; Veh 23.3 \(\pm\) 0.1 h; Pin-X 23.1 \(\pm\) 0.1 h; and Mel 23.4 \(\pm\) 0.1 h. There were no statistically significant differences among the periods of Sham and Pin-X chicks or among the periods of Veh and Mel chicks. This rhythm was used as a phase marker for recording ERGs in DD.

Raw ERG data from an intact chick. The effect of changing light stimulus intensity and time of day was immediately apparent in the raw ERG data. Actual ERG traces taken at midday and midnight at various light stimulus intensities from a single representative intact chick are shown in Fig. 2. As intensity decreased, a- and b-wave amplitude decreased, and a- and b-wave implicit time increased at both times of day. At midday, at most light stimulus intensities, the a- and b-wave amplitudes were higher and implicit times were shorter than at midnight. Each trace is an average of 5 ERG recordings. ZT, zeitgeber time.

Fig. 2. Electroretinogram (ERG) recordings of a single intact chick in LD. ERG traces recorded at midday and midnight at various light stimulus intensities recorded are shown. As intensity decreased, a- and b-wave amplitude decreased, and a- and b-wave implicit time increased at both times of day. At midday, at most light stimulus intensities, the a- and b-wave amplitudes were higher and implicit times were shorter than at midnight. Each trace is an average of 5 ERG recordings. ZT, zeitgeber time.

Experiment 1: ERG rhythms in Sham and Pin-X chicks in LD and DD. In LD, a- and b-wave response amplitudes of intact (data not shown), Sham (Fig. 3, A and B), and Pin-X (Fig. 3, C and D) chicks increased in a second-order fashion with increasing light stimulus intensity. The a- and b-wave response amplitudes were higher during the day than during the night only at several of the highest intensities. The intensity-response implicit time relationships of both the a- and
Fig. 3. Intensity-response amplitude and intensity-response implicit time of Sham and Pin-X chicks in LD. The a- and b-wave intensity-response amplitude of Sham (A and B) and Pin-X (C and D) chicks increased with increasing light stimulus intensities in a second-order fashion. The a- and b-wave intensity-response implicit time of Sham (E and F) and Pin-X (G and H) chicks increased in a linear fashion with increasing light stimulus intensities.
b-waves for intact (data not shown), Sham (Fig. 3, E and F), and Pin-X (Fig. 3, G and H) chicks were linear such that implicit time increased with decreasing light stimulus intensity. Although ERG a-wave implicit time was higher during the night than during the day at all light stimulus intensities (Fig. 3E), b-wave implicit time was only rhythmic at the lower light intensities, although still higher during the night than during the day (Fig. 3F).

In LD, there was a daily rhythm in a- and b-wave amplitude (Fig. 4, A and B) in Sham and Pin-X chicks such that a- and b-wave amplitudes were higher during the day than during the night. The a- and b-wave implicit times (Fig. 4, C and D) of both Sham and Pin-X chicks were also rhythmic such that implicit times were shorter during the day than during the night. There was a statistically significant rhythm in a-wave sensitivity (Fig. 4E) of both Sham and Pin-X chicks, whereas there was no difference in b-wave sensitivity (Fig. 4F). Note that a lower value for median efficacious intensity (EI₅₀) reflects higher sensitivity. *Significant difference from values obtained during the day from Sham birds. #Significant difference from values obtained during the day from Pin-X birds.
there was no rhythm in b-wave sensitivity (Fig. 4F) of either group. The sensitivity of the a-wave was such that both Sham and Pin-X chicks were more sensitive to light during the night than during the day. Note that low EI50 values correspond to high sensitivities. There was no statistically significant difference between Sham and Pin-X chicks in either a- or b-wave amplitude, implicit time, or sensitivity in LD.

In DD, the intensity response amplitude and implicit time relationships for both the a- and b-waves of Sham and Pin-X chicks (data not shown) were similar to those observed in LD (Fig. 3). Neither Sham nor Pin-X chicks expressed rhythmic a-wave amplitude, nor did they express rhythms in a- or b-wave sensitivity in DD (data not shown). However, Pin-X partially abolished several rhythms that persisted in Sham birds in DD. Whereas rhythmicity persisted in the b-wave amplitude of Sham chicks such that amplitude was high during the subjective day, this rhythm was lost in Pin-X chicks (Fig. 5A). Sham chicks in DD also retained rhythmicity in a- and b-wave implicit times (Fig. 5, B and C) such that implicit time was shorter during the subjective day than during the subjective night. Pin-X chicks retained rhythmicity in a-wave implicit time with the same phase (Fig. 5B), although at a very low amplitude. No rhythmicity in b-wave implicit time was observed in Pin-X birds in DD (Fig. 5C).

Experiment 2: ERG rhythms in Veh and Mel chicks in LD and DD. RIA for melatonin on serum taken from Veh and Mel chicks in LD (Fig. 6A) and in DD (Fig. 6B) indicated that, in LD, Veh chicks expressed a daily rhythm in serum melatonin content such that melatonin was high during the night and low during the day. Mel chicks did not have a statistically significant daily rhythm in serum melatonin, probably due to the high SE at ZT6, although the overall level of melatonin appears to be higher in Mel chicks than in Veh chicks both at midday and midnight. In DD, both Veh and Mel chicks expressed circadian rhythms in serum melatonin content such that melatonin was statistically higher during the subjective night than during the subjective day. The overall level of melatonin, however, was higher in Mel chicks than in Veh chicks at midday and midnight.

In LD, both Veh and Mel chicks exhibited daily rhythms in a- and b-wave amplitude (Fig. 7, A and B), a- and b-wave implicit time (Fig. 7, C and D), and a-wave sensitivity (Fig. 7E), whereas neither Veh nor Mel chicks were rhythmic in b-wave sensitivity (Fig. 7F). The a- and b-wave amplitudes were higher, a- and b-wave implicit times were shorter, and a-wave sensitivity was lower during the day than during the night. There was no statistically significant difference between Veh and Mel chicks in either a- or b-wave amplitude, implicit time, or sensitivity of the visual response in LD.

In DD, neither Veh nor Mel chicks were rhythmic in a-wave amplitude or in a- or b-wave sensitivity (data not shown). The rhythm of b-wave amplitude, however, persisted in Veh chicks but was abolished in Mel chicks (Fig. 8A). Furthermore, the rhythms of a- and b-wave implicit time (Fig. 8, B and C) persisted in Veh chicks but were also abolished in Mel chicks. The rhythms that persisted had a similar phase angle as in LD; amplitude was higher, and implicit time was shorter during the subjective day than during the subjective night. Although the b-wave amplitude of Mel chicks was suppressed to a low nighttime level during the
subjective day, a- and b-wave implicit times were at intermediate levels at both midsleep day and midsleep night.

**DISCUSSION**

The pineal gland and its hormone melatonin at least partially contribute to the circadian regulation of ERG (Figs. 5 and 8), but, unlike the situation in passerine birds where they are crucial (11, 14), they have little or no role in rhythms of behavioral feeding activity (Fig. 1). Pinealectomy abolished DD rhythms of b-wave amplitude and implicit time, whereas the rhythm of a-wave implicit time persisted in DD after surgery, albeit with a lower amplitude (Fig. 5). In Sham birds, rhythms of b-wave amplitude and a- and b-wave implicit time persisted in DD. In contrast to the situation in DD, the surgery had no effect on rhythms of ERG a- and b-wave amplitude, implicit time, or sensitivity in LD (Figs. 3 and 4). The a- and b-wave amplitude was higher, a- and b-wave implicit time was shorter, and a-wave sensitivity was lower during the day than during the night in both Pin-X and Sham birds. These data suggest that the pineal gland, presumably via its circadian secretion of the hormone melatonin, influences retinal rhythmicity.

Pinealectomy, however, does not remove all of the melatonin produced by chicks. Melatonin is also synthesized in the retinas in a daily and circadian fashion (1, 2, 4, 16). Because pinealectomy did not fully abolish ERG rhythms, it was thought that retinal melatonin might be involved in regulation of ERG rhythms. To test for this possibility, continuous high doses of melatonin were delivered to whole animals via Silastic capsules containing melatonin. Similar to the situation with pinealectomy, exogenous melatonin also had no effect on ERG rhythms in LD (Fig. 7), but rhythms were abolished completely in Mel chicks maintained in DD (Fig. 8). b-Wave amplitude was suppressed during the subjective day to low nighttime levels, and a- and b-wave implicit times were at intermediate levels during both the subjective day and the subjective night. In DD, Veh chicks retained rhythmicity in b-wave amplitude and in a- and b-wave implicit times. Thus continuous administration of exogenous melatonin, even though the administration did not completely block serum melatonin rhythms, abolished all DD rhythms in ERG.

These results suggest a regulatory mechanism for a daily and circadian Purkinje shift model of avian retinal function (31 and J. J. McGoogan, W. Q. Wu, and V. M. Cassone, unpublished observations). In this model, high amplitude, short implicit time, and low sensitivity during the day reflect cone activity, and low amplitude, long implicit time, and high sensitivity during the night reflect rod activity. We hypothesize that this Purkinje shift of cone and rod activity is regulated on a daily and circadian basis at least partially by melatonin. However, removal of pineal melatonin or administration of exogenous melatonin is sufficient to abolish the Purkinje shift rhythm in DD, but not in LD. It is important to point out here that blood melatonin does enter the chick retina and increases retinal serotonin content and catabolism (10). Therefore, there must be some other component(s) of the regulatory mechanism that is sufficient to drive the rhythm in LD but not in DD without the rhythm of melatonin.

This other component regulating retinal rhythms may be the photoreceptors themselves, which are known to contain circadian oscillators (18), especially since it is the rhythm of a-wave implicit time, a reflection of photoreceptor activity, that is preserved after Pin-X (Fig. 5). Alternatively, dopaminergic elements of the neural retina (34) may contribute to retinal rhythmicity. Retinal dopamine content is rhythmic on a circadian basis such that vitreal dopamine concentrations are higher during the day than during the night in LD and DD (2). Furthermore, dopaminergic agonists inhibit melatonin biosynthesis in the chick retina via D<sub>2</sub>
dopamine receptors in a phase-dependent fashion (2, 34), just as nocturnal melatonin inhibits dopamine release (25). Indeed, a mutually inhibitory loop of melatonin and dopamine has been proposed in the avian retina (1, 2, 25, 31). It may be that either the rhythm of melatonin or the rhythm of dopamine is sufficient to drive the Purkinje shift and perhaps other related retinal rhythms in LD but that both are necessary to drive the rhythm in DD.

Perspectives

On a broader scale, the present data suggest that our model for avian circadian organization (6, 11) must be modified and extended (Fig. 9). The “neuroendocrine loop” model (6) and the related “internal resonance” model (14) for avian circadian organization originally proposed that each component of the system is damped circadian oscillators that maintained rhythmicity via...
their mutual, inhibitory interactions. On one side, the vSCN is a circadian oscillator that is active primarily during the day in LD and subjective day in DD (19). This oscillator is innervated by the retinas via a retinohypothalamic tract and is presumably entrained by the LD cycle directly. On the other side, the pineal gland and retinas are also damped circadian oscillators whose mutual output, the secretion of melatonin, occurs during the night in LD and subjective night in DD. These oscillators are directly photoreceptive and may be entrained independently. In the presence of an LD cycle, each oscillator can sustain rhythmicity, because each is directly entrained. However, in the absence of an LD cycle, each oscillator damps gradually to arrhythmicity, unless it receives inhibitory input from one or another of the system's oscillators. During the day, the vSCN, via a multisynaptic pathway including the sympathetic superior cervical ganglia, inhibit pineal melatonin release and prevent damping via release of norepinephrine during the subjective day (10, 32). It is not known whether the retinas are regulated similarly. However, an analogous loop may reside within the retinas themselves, with dopamine acting in norepinephrine's place (25, 34). During the night, melatonin released by the pineal gland and, in some species, the retinas inhibit vSCN activity acutely (7) and synchronize a daily rhythm of 2-DG uptake (20).

As stated above, feeding activity was measured and used as an independent assessment of phase. In both LD and DD, chicks were rhythmic in feeding activity such that birds were active during the day in LD and ran free in DD with a short free-running period. There was no statistically significant difference between the periods of feeding activity of Sham and Pin-X chicks and Veh and Mel chicks. This insensitivity of feeding rhythms to either pinealectomy or exogenous melatonin has been reported previously in European starlings (15) and domestic pigeons (12). The simultaneous assessment of rhythmicity of two clock outputs, feeding activity and retinal visual function, while manipulating one clock component, melatonin, suggests that, although system oscillators are coupled together such that they function as a unit, each oscillator drives different outputs (Fig. 9). Although pinealectomy severely disrupts and exogenous melatonin abolishes
rhythms of retinal visual function as measured by ERG in DD, neither treatment has any effect on the rhythm of feeding activity. This suggests that, although retinal and pineal melatonin are involved in driving ERG outputs, they are not involved in driving the feeding activity output. It is likely that the clock located in the vSCN or elsewhere drives the feeding activity rhythm. If this is the case, we might expect vSCN lesion to produce the opposite result: the feeding activity rhythm would be disrupted or abolished, and ERG rhythms would be unaffected.

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