Circadian Rhythm of ERG in 
Iguana iguana: Role of the Pineal

Manuel Miranda-Anaya, Paul A. Bartell, Shin Yamazaki, and Michael Menaker

Department of Biology and National Science Foundation, Center for Biological Timing, 
University of Virginia, Charlottesville, VA 22903, USA

Abstract  In green iguanas, the pineal controls the circadian rhythm of body temperature but not the rhythm of locomotor activity. As part of a program to investigate the characteristics of this multioscillator circadian system, the authors studied the circadian rhythms of the electroretinographic response (ERG) and asked whether the pineal gland is necessary for the expression of this rhythm. ERGs from a total of 24 anesthetized juvenile iguanas were recorded under four different conditions: (a) complete darkness (DD), (b) dim light-dark cycles (dLD), (c) constant dim light (dLL), and (d) pinealectomized in DD. Results demonstrate that the b-wave component of the ERG shows a very clear circadian rhythm in DD and that this rhythm persists in dLL and entrains to dLD cycles. The ERG response is maximally sensitive during the subjective day. Pinealectomy does not abolish the circadian rhythm in ERG, demonstrating that the oscillator responsible for the ERG rhythm is located elsewhere.

Key words  circadian rhythms, electroretinogram, Iguana iguana, pineal gland

Circadian rhythms enable organisms to segregate specific physiological and behavioral functions to the times at which they are most adaptive. Circadian rhythmicity is commonly observed in many aspects of visual function and is often assayed by measuring the elicited electroretinogram (ERG). Circadian rhythms of ERG have been reported for many invertebrates (Page and Larimer, 1974; Larimer and Smith, 1981; Barlow, 1983; Wills et al., 1985; Block et al., 1993; Yamazaki et al., 1995), as well as fish (Nussdorf and Powers, 1988; McMahon and Barlow, 1992), birds (Lu et al., 1995; Kelly et al., 1996; Manglapus et al., 1998), reptiles (Fowlkes et al., 1984), and mammals (Brandenburg et al., 1981). Circadian rhythms in retinal sensitivity are of particular interest in the study of vertebrate circadian organization because retinal innervation of the suprachiasmatic nucleus (SCN) is an important pathway for input of photic information to the circadian system in birds (Cassone and Moore, 1987), reptiles (Janik et al., 1994; Kenigfest et al., 1997), and mammals (Moore, 1973).

Circadian systems composed of multiple autonomous circadian oscillators that regulate diverse outputs within the same organism have been described in birds (Cassone and Menaker, 1984), reptiles (Underwood, 1977, 1981, 1983; Tosini and Menaker, 1998), amphibians (Harada et al., 1998), and mammals (Tosini and Menaker, 1996a). Understanding the organization of such systems, specifically how the component oscillators interact to produce a coordinated adaptive temporal structure, is a major problem in circadian biology. An important first step is determining if there is segregated control, and, if so, which oscillators control which overt rhythms. Because the green iguana possesses multiple circadian photoreceptors, multiple autonomous circadian oscillators, and several separately measurable circadian rhythms (Tosini and Menaker, 1996b, 1998), it is an attractive
organism for such analysis. We asked if changes in visual sensitivity could be added to this animal’s repertoire of circadian outputs and whether, if rhythms of ERG exist, they are regulated (as is the rhythm of body temperature) by the pineal gland.

An ERG elicited from a typical diurnal lizard is composed of several components (Meneghini and Hamasaki, 1967). The normal response to the onset of a short duration light pulse (<500 ms) is an initial negative corneal deflection (a-wave) produced by the photoreceptors. This is followed by a rapidly rising positive potential (b-wave), which is the result of extracellular current established around Müller cells of the inner retina (Miller and Dowling, 1970; Dowling, 1987) and reflects the activity of the ON bipolar cells. The d-wave is an additional positive component originating as a response of the OFF bipolar cells during the off response (Stockton and Slaughter, 1989). Comparative information about the circadian properties of evoked ERGs from different species of lizards is sparse. In the nocturnal lizard Gecko gecko, a circadian rhythm in the b-wave component of the ERG with the highest amplitude during the early subjective night has been reported by Fowlkes et al. (1987). In the diurnal iguanid lizard Anolis carolinensis, a circadian rhythm in the b-wave has also been measured with the highest amplitude observed during the middle of the subjective day (Fowlkes et al., 1984; Shaw et al., 1993).

Although circadian rhythms of ERG amplitude have been widely used to measure circadian rhythms in the eye, the location of the oscillators responsible for this rhythm may vary among the vertebrates. In quail, the rhythm persists after cutting the optic nerve, which suggests that the oscillator is located in the eye in this animal (Konishi and Homma, 1984). In the lizard A. carolinensis, removal of the pineal gland abolishes the circadian rhythm of the b-wave component of the ERG (Shaw et al., 1993). In the rabbit, removing the superior cervical ganglion, which supplies sympathetic input to many structures in the head including the pineal, abolishes the circadian rhythm of ERG (Brandenburg et al., 1981).

In vertebrates, the pineal gland is the main site of melatonin synthesis and release, although in some species, such as the Japanese quail, the retinai contribute significantly to the profile of melatonin circulating in the blood (Underwood and Siopes, 1984). The administration of exogenous melatonin has been shown to decrease the amplitude of the ERG response in domestic fowl (Lu et al., 1995), rabbits (Textorius and Nilsson, 1987), and even humans (Emser et al., 1993). Melatonin administration appears to be most effective when applied intramuscularly or intraperitoneally, as opposed to intracovertly. This suggests that in these organisms (as well as in A. carolinensis), nonretinal melatonin may influence the ERG and that in some cases it may be exerting its effects on a more centrally located structure, possibly the SCN, rather than on the eye itself. Our results demonstrate that the pineal of Iguana iguana does not contain the circadian oscillator responsible for the rhythm in ERG, but they suggest that pineal melatonin may modulate this rhythm.

MATERIALS AND METHODS

Animal Maintenance

Juvenile green iguanas, I. iguana (60-90 g) were obtained from Glades Herpetological Supply (Fort Myers, FL) and housed in plastic cages lined with pine shavings. The cages were contained within a large wooden enclosure (130 x 70 x 40 cm.), with heat provided by a thermal pad placed underneath the cages. The temperature was maintained at 28 ± 2 °C and a 12-h light:12-h dark cycle (LD 12:12) was provided by a fluorescent lamp (20 W, Philips). The iguanas were fed a mixture of Zu-Pream marmoset chow and vegetable greens thrice weekly with water provided ad libitum.

ERGs were recorded for at least 3 days from continuously anesthetized animals. Long-term anesthesia was provided by intraperitoneal injections of urethane (2.5 g/Kg) at the beginning of each experiment. To provide additional analgesia, a solution of 10% procaine in physiological saline was topically administered to the cornea. The lids of the eyes were then sutured open and the iguana was transferred to a Styrofoam box and placed on a copper plate (15 x 10 cm). The head was held in place by padded clamps. A recirculating water bath (Lauda MGW) was connected to channels within the copper plate to maintain a constant deep body temperature of 28 °C.

Recording Procedure

ERGs were obtained using a bare loop (aprox. 5 mm diam.) of Teflon-coated platinum-iridium wire (A.M. Systems, Inc. Carlsborg, WA; 0.07 cm bare). The active electrode was positioned on the surface of the cornea,
and the reference electrode was inserted subcutaneously in the front of the head. The cornea was then covered with a high-viscosity clear silicone (Dow Corning, Midland, MI; 200 Fluid 60,000 CS). Electrical activity was amplified using a wide-band EEG-AC preamplifier (Grass mod. 7P5 band pass filter 3-1500 Hz) coupled to a DC driver (Grass mod. 7DA), and recorded on a polygraph (Grass mod. 79D).

A 250 ms light stimulus of 50 µW cm⁻² (log I = –1.7) was provided by illuminating one end of a fiber optic bundle with a halogen lamp (Dolland Jenner mod 4715 MS). The light intensity was regulated with interference filters. An electronically controlled shutter delivered the light pulse to the fiber optic bundle automatically every 15 min. The other end of the fiber optic bundle was placed approximately 3 mm from the surface of the eye.

During the several days of ERG recording, light for light-dark (dLD) cycles or constant dim light (dLL) was provided by an incandescent light bulb (GE; 12 W, 12 V) located 75 cm above the animal. The lamp was arranged to provide a light intensity of 0.5 µW cm⁻² at the level of the iguana’s head. To avoid changes in the ambient temperature during the light period, we used a low-speed fan to keep the area around the bulb ventilated. Light intensities were measured using a photometer (Graseby Optronics, Model 350; Orlando, FL). The temperature was monitored with a Tele Thermometer (Yellow Springs Instruments, mod 44TB, Yellow Springs, OH) with the probe located directly under the iguana’s abdomen.

Pinealectomy

Surgery was performed using a modification of the procedure reported by Tosini and Menaker (1996b). To avoid phase shifts, all surgical procedures were done during the animal’s day (6-8 h after lights on). The animals were first cooled in a refrigerator (4 °C) until immobile and then embedded in crushed ice. A solution of procaine (10% in saline) was administrated locally at the incision site. A flap of skin above the skull was removed, and a small hole (1.8 mm diameter) was drilled in the skull above the pineal using a dental drill. The exposed dura was removed, the meninges cut, and the pineal was grasped and removed with a pair of fine forceps. The pineal was visually inspected after removal to make sure it remained intact. To reduce bleeding during surgery, a solution of norepinephrine (4 mM) was applied topically to the wound. The wound was then packed with Gelfoam and covered with cyanoacrylate tissue adhesive (Nexband). The iguanas were warmed to room temperature using a heating pad and 2 h later transferred back to their original cages. The ERG recordings were done at least 3 days after pinealectomy. After recording ERGs from the iguanas, they were perfused and the pineal area was visually inspected to make certain that no obvious bits of pineal tissue remained.

Plasma Extraction and Radioimmunoassay

Blood samples (200 µl) were obtained with heparinized syringes via cardiac puncture as described by McDonald (1976). Samples from 3 control and 5 pinealectomized iguanas were collected at midday and midnight. The samples were transferred to heparinized tubes and centrifuged at 4000 RPM/45 min. The plasma was then transferred to another tube and stored at –80 °C until assayed. The melatonin assay was performed using a modification of the method validated for iguanas by Tosini and Menaker (1996b) and based on the protocols developed by Rollag and Niswender (1976). Fifty µl of plasma from each blood sample were extracted in chloroform (2 ml), washed with 0.1 M sodium carbonate buffer (pH 10.25), and then with milli-Q water. The remnant was dried under nitrogen gas and resuspended in 0.5 ml of phosphate buffer with 0.9% NaCl and 1% gelatin and assayed by radioimmunoassay using the melatonin antiserum R1055.

Protocols

Animals were tested independently under one of four different conditions: dim light-dark cycle (dLD 12:12, n = 6); constant dim light (dLL, n = 6), intact animals in constant darkness (DD, n = 7), or pinealectomized animals in constant darkness (Pin-X DD; n = 5).

Data Analysis

The a- and b-wave amplitudes were measured by hand and converted to µV using the calibration reference in each experiment. The a-wave amplitude was observed as a corneal negative deflection that appears immediately after the onset of light from each pulse and was measured from the projected baseline. The b-wave amplitude was measured as the voltage difference between the trough of the a-wave and the peak of the b-wave (Fig. 1). A negative wave is present after
the b-wave, which was considered an artifact of the low cutoff filter because it does not correspond with any component described in the ERG for the green iguana (Meneghini and Hamasaki, 1967). Finally a d-wave component, expressed as a low-amplitude positive deflection, is seen immediately after the light stimulus is turned off. This last wave was not measured since it is a slow wave of low amplitude and is easily masked by the background noise. An iterative, coupled, fast Fourier transform–nonlinear least squares estimation method was used to determine the circadian period (Developed by Dr. M. Straume, NSF Center for Biological Timing, University of Virginia; see Plautz et al., 1997). Circadian periods meeting the 95% level of confidence were considered acceptable. The parameters were subjected to an unpaired, unequal variances t-test, and the variances were analyzed using an F test to determine significance.

RESULTS

Figure 2 shows the variation in the waveform amplitudes of the ERGs from one dark-adapted animal elicited at six different light intensities. The highest intensity used was considered log \( I = 0 \) (2000 \( \mu W \text{ cm}^{-2} \)). The amplitude at each intensity appears quite different between midday and midnight, while the threshold intensity necessary to elicit a reliable response was log \( I = -2.8 \) (3 \( \mu W \text{ cm}^{-2} \)) at both times. The amplitude of the b-wave is clearly different when comparing midday and midnight responses at log \( I = -1.7 \) and higher intensities. The difference in amplitude is approximately 30% between these two sampling times; this difference is proportional at higher intensities (log \( I = -0.7 \) and Log \( I = 0 \)). At Log \( I = -0.7 \), a difference in the amplitude of the a-wave is clearly seen; however, circadian differences in a-wave amplitude are not consistent in most of the records. Furthermore, the a-wave is not always measurable, even when using the same stimulus intensity under the same conditions.

In all of the records obtained, the amplitude of the b-wave shows a circadian rhythm. However, the amplitude of the a-wave demonstrated a circadian period within the 95% level of confidence in only a few records. Furthermore, this period was not the same as the period of the b-wave in the same subject, a difference that might well be the result of the poor quality of the a-wave data.
The Amplitude of the b-Wave in the Iguana ERG Is Modulated by a Circadian Oscillator in Constant Darkness

Figure 3 shows the circadian rhythm of ERG amplitude from 2 different animals in DD. In Figure 3a, a 6-day record shows only the rhythm of the b-wave. The gap at the end of the fourth day represents missing data resulting from equipment failure. The period is 23.4 h, and the peak of activity is at projected midday (MD in figures) on the first day of the record. A similar rhythm is shown in Figure 3b (τ = 22.56 h). The waveform of the circadian rhythm is variable under DD conditions; however, most frequently there is a sharp peak in b-wave amplitude.

Expression of the Entrained Rhythm in b-Wave Amplitude under dLD Conditions

Figure 4a shows a typical ERG record from dLD conditions. The period of the rhythm (24.05 h) suggests entraining to the light-dark cycle, and the maximum amplitude is observed at midday. The amplitude of the b-wave clearly increases before lights on and decreases before lights off (0700 and 1900 h, respectively), an indication of the animal’s anticipation of the changing conditions. Figure 4b shows an example in which the light cycle was delayed 5 h; after four cycles of treatment, the rhythm regains its normal phase relationship to the light cycle. Here it is possible to observe transients in the phase where the maximum amplitude appears. On the fifth day the rhythm seems to be entrained.

The Circadian Rhythm of ERG Persists in Constant Dim Light

The circadian rhythm in b-wave amplitude persists under dLL conditions, as demonstrated in the two records shown in Figure 5. The value of the free-running period (τ = 25.87 ± 0.52 h) is significantly longer than that in constant darkness (τ = 23.31 ± 0.54 h, p < 0.05). Figure 5a shows a circadian rhythm over five continuous cycles with the rhythm gaining robustness in the last three cycles. In Figure 5b, a 3-day-long record shows a clear rhythm in the b-wave amplitude; the a-wave, although measurable, is not statistically rhythmic. In dLL conditions, the periods of the rhythms were longer than 24 h in all the animals tested.
Pinealectomy Does Not Abolish the Circadian Rhythm of the b-Wave Amplitude

Figure 6 shows circadian rhythms of b-wave amplitude from two different pinealectomized iguanas in constant darkness. In Figure 6a, a 6-day-long recording from a pinealectomized iguana shows a clear circadian rhythm of the b-wave amplitude with $\tau = 25.42$ h. In Figure 6b, the rhythm of the b-wave is robust ($\tau = 25.44$ h) while the a-wave does not show a significant circadian rhythm and is absent from many samples during the last day. Pinealectomy affects the expression of the amplitude of the b-wave. The highest amplitude observed in each cycle in pinealectomized iguanas ($86.28 \pm 6.12 \mu V$) and that observed in intact animals ($72.54 \pm 3.5 \mu V$) are significantly different in DD ($t$-test, $p < 0.05$); however, there are no differences in amplitude during the subjective night.

Figure 7 shows the average circadian periods (± SE) of the b-wave under the different conditions tested. Differences in period length were observed between DD and dLL conditions for intact animals ($t$-test, $p < 0.05$). Although the average circadian periods of ERGs from intact and pinealectomized iguanas were not different when measured in DD, there was a significant difference in period length variance ($F$ test, $p < 0.05$).

To ensure the efficacy of our pinealectomies, we measured circulating levels of melatonin in the animals’ plasma. The results from 5 pinealectomized and 3 control iguanas held in 12:12 LD cycles were compared. In control animals, significant variation ($p < 0.01$) in the levels of circulating melatonin was observed between midday ($217.75 \pm 9.82$ pg/ml) and midnight ($512 \pm 11.09$ pg/ml). There were no significant differences between samples taken during midday ($228.5 \pm 25.8$ pg/ml) and midnight ($240 \pm 1.15$ pg/ml) in pinealectomized animals, nighttime levels of melatonin in the blood were significantly different ($p < 0.01$) between control and pinealectomized animals. Pineal glands were also found to be absent during visual inspections of the perfused animals’ brains after the recordings were completed.

Rhythmic variations in concentration of circulating melatonin in intact adult iguanas and an absence of melatonin rhythms in pinealectomized iguanas have been previously reported by Tosini and Menaker (1996a). In the present study, the levels of nighttime melatonin in pinealectomized iguanas were found to be slightly higher than those reported for adults. This difference may be due to the younger age of the animals in the present study. The presence of circulating melatonin in pinealectomized iguanas demonstrates that it is produced by nonpineal structures, although these extra pineal sources are not responsible for the circadian rhythm of circulating melatonin.
DISCUSSION

The amplitude of the b-wave component of the lateral eye ERG of juvenile green iguanas is modulated by a circadian oscillator. This rhythm could be measured in all the animals tested; its maximum amplitude is during the day in the presence of LD cycles and during the projected midday of the first day of recording in constant conditions. This corresponds with the peak in visual sensitivity observed in the diurnal lizard *Anolis* (Fowlkes et al., 1987). The iguana retina is composed almost exclusively of cones (Meneghini and Hamasaki, 1967), and therefore it is possible that it does not undergo the shift in dominance from cones to rods that is seen in some other diurnal animals, such as quail, upon dark adaptation (Manglapus et al., 1998).

A few iguanas displayed rhythmicity in the amplitude of the a-wave component of the ERG. A low percentage of cases of rhythmicity in a-wave amplitude have also been reported in ERGs from *A. carolinensis* (Fowlkes et al., 1987). A complete absence of rhythmicity in the a-wave amplitude has been reported in the domestic fowl (Lu et al., 1995), but rhythmicity is observed with varying amplitudes in the a-wave of the Japanese quail (Manglapus et al., 1998). These authors suggest that the absence of rhythmicity in the a-wave amplitude may sometimes be the result of masking by the b-wave. At the intensity we used to elicit ERGs, the a-wave has a low amplitude and thus any changes in that amplitude are not easily observed. Higher intensities of light produce bigger a-wave amplitudes, but in most cases rhythmicity is still not clear (data not shown) while the b-wave amplitude often becomes saturated.

The pineal gland is not necessary for circadian rhythmicity of b-wave amplitude in the green iguana. All of the pinealectomized animals retained their circadian rhythms of b-wave amplitude; however, the maximum amplitude of the ERG response and the variability in the length of the free-running periods was different from control animals. These results are different from those reported for the iguanid lizard *A. carolinensis*. In that animal, the rhythm in b-wave amplitude is abolished by removal of the pineal gland. Our data demonstrate that the oscillator that generates this rhythm of retinal sensitivity in the green iguana is not located in the pineal gland.

Pinealectomy in young iguanas abolishes the rhythm of circulating melatonin as it does in adults (Tosini and Menaker, 1996b). However, pinealectomy does not completely eliminate melatonin from the blood, suggesting that nonpineal structures can produce this indolamine. The finding of higher levels of melatonin in juvenile green iguanas than in adults suggests that its production may vary during ontogeny.

The effects of pinealectomy on locomotor activity of lizards range from a complete loss of circadian

Figure 7. The average values (±SE) of the periods of the circadian rhythm in b-wave amplitude elicited from iguanas under each experimental condition. There are significant differences in the lengths of the free-running periods between DD and constant dim light (dLL) conditions ($p < 0.05$). There are also differences in the variance between intact animals and pinealectomized animals kept in constant darkness ($p < 0.05$).
rhythms of locomotor activity in *Anolis* (Underwood, 1983), to changes in the free-running periods in *Sceloporus* (Underwood, 1977, 1981) and *Podarcis* (Innocenti et al., 1996), to no detectable effects on locomotor behavior in *Dipsosaurus* (Janik and Menaker, 1990). The limited data available for the ERG rhythms of lizards indicate a similar pattern; pinealectomy abolishes ERG rhythmicity in *Anolis*, but fails to do so in *Iguana*, although it does affect the amplitude of the rhythm, suggesting that pineal melatonin may modulate retinal sensitivity.

Melatonin has been demonstrated to inhibit dopaminergic systems in the retinas of vertebrates and to modulate the transmission of light signals to the brain (reviewed in Dubocovich et al., 1995). Autoradiographic studies using 125I labeled iodo-melatonin, a melatonin analogue, demonstrate that there are melatonin binding sites in the innerplexiform layer of the *Anolis’s* retina (Weichmann and Wirsig-Weichmann, 1994). Interestingly, the b-wave component of the ERG is in part the result of a light-induced potassium current from within that retinal layer (Miller and Dowling, 1970; Stockton and Slaughter, 1989). The retina of the lateral eye of *I. iguana* contains a circadian oscillator that controls melatonin synthesis. Melatonin production under constant darkness in vitro is rhythmic, with maximum melatonin synthesis occurring during the middle of the projected night (Tosini and Menaker, 1998). Although the circadian rhythm in b-wave amplitude is clearly not driven primarily by pineal melatonin, it may be driven by melatonin rhythmically produced in the retina itself. Alternatively, the ERG rhythm could be the consequence of any number of rhythmic processes within the retina or the oscillator driving the rhythm could be located elsewhere (possibly in the SCN) and act on the retina via neural or humoral pathways.

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