

Circadian Rhythm of Iguana Electroretinogram: The Role of Dopamine and Melatonin

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Abstract The amplitude of the b-wave of an electroretinogram (ERG) varies with a circadian rhythm in the green iguana; the amplitude is high during the day (or subjective day) and low during the night (or subjective night). Dopamine and melatonin contents in the eye are robustly rhythmic under constant conditions; dopamine levels are high during the subjective day, and melatonin levels are high during the subjective night. Dopamine and melatonin affect the amplitude of the b-wave in an antagonistic and phase-dependent manner: dopamine D2-receptor agonists injected intraocularly during the subjective night produce high-amplitude b-waves characteristic of the subjective day, whereas melatonin injected intraocularly during the subjective day reduces b-wave amplitude. Sectioning the optic nerve abolishes the circadian rhythms of b-wave amplitude and of dopamine content. The results of this study suggest that in iguana, a negative feedback loop involving dopamine and melatonin regulates the circadian rhythm of the ERG b-wave amplitude that is at least in part generated in the brain.

Key words electroretinography, circadian, dopamine, melatonin, optic nerve, iguana

Many behavioral and physiological changes in animals are regulated by the circadian system, which is responsive to daily changes in the levels of illumination. The circadian system of vertebrates consists of multiple oscillators and photoreceptors located in different tissues and their interactions, with the outputs from different oscillators directly regulating different aspects of physiology and behavior (Menaker and Tosini, 1995; Yamazaki et al., 2000).

The retinas of vertebrates contain circadian oscillators that mediate physiological processes involved in

visual sensitivity (Besharse et al., 1988; Herzog and Block, 1999; Li and Dowling, 1998). Outputs of these oscillators include melatonin and dopamine. Melatonin is synthesized rhythmically in cultured retinas of lamprey (Menaker and Tosini, 1995), fish (Cahill, 1996), amphibians (Cahill and Besharse, 1995; Alonso-Gómez et al., 2000), reptiles (Tosini and Menaker, 1998), and mammals (Tosini and Menaker, 1996a), and a circadian rhythm of ocular melatonin has been reported in birds (Underwood and Siopes, 1984; Adachi et al., 1998). Melatonin is involved in the

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activation of disk-shedding and cone movement in *Xenopus* (Besharse et al., 1988) and decreases b-wave amplitude of the electroretinogram (ERG) in birds (Lu et al., 1995; McGoogan and Cassone, 1999) and humans (Emser et al., 1993). It inhibits dopamine release from amacrine cells (Dubocovich, 1983), suggesting that it may influence dopamine-dependent mechanisms of light and dark adaptation.

Dopamine is an abundant neurotransmitter in the retina (Dowling, 1987; Nguyen-Legros, 1996), and circadian rhythms in ocular content of dopamine have been described in fish (McCormack and Burnside, 1993), birds (Adachi et al., 1998), and mammals (Doyle et al., 1999). Dopamine affects circadian rhythms of melatonin synthesis, disk shedding, rod elongation, and retinal pigment epithelium dispersion, as well as cone-rod inputs in the retina of *Xenopus* (Besharse et al., 1988; Cahill and Besharse, 1991; Krizaj et al., 1998). Dopamine also regulates the circadian rhythm of visual sensitivity in fish (Li and Dowling, 2000) and quail (Manglapus et al., 1999).

The ERG measures the global electrical response of the retina to a flash stimulus and is a useful tool to evaluate visual function (Dowling, 1987). ERG b-wave amplitude depends on postphotoreceptor electrical activity and displays a circadian rhythm in constant conditions in fish (Li and Dowling, 2000), birds (Lu et al., 1995; Manglapus et al., 1998; McGoogan and Cassone, 1999; Wu et al., 2000), reptiles (Fowlkes et al., 1987; Shaw et al., 1993; Miranda-Anaya et al., 2000), and mammals (Brandenburg et al., 1983). In many of these studies, either melatonin or dopamine has been proposed as a regulator of rhythms of ERG b-wave amplitude.

In the green iguana, an oscillator of unknown location regulates the circadian rhythm of ERG b-wave amplitude. Dopamine content in the eye peaks during the subjective day, whereas melatonin content peaks during subjective night. Intraocular injections of the dopamine D2 receptor agonist quinpirole increase the amplitude of the b-wave during the subjective night, whereas melatonin injections decrease it during the subjective day. Depleting the retinas of dopamine abolishes the circadian rhythm of ERG. Because sectioning the optic nerve abolishes the rhythm of retinal dopamine content, whereas the rhythm of melatonin synthesis persists in cultured retinas, our data suggest that the rhythm of dopamine content and the rhythm of melatonin synthesis are under the control of different circadian oscillators. We propose that the circadian rhythm of b-wave amplitude in the iguana is regu-

lated by the interaction of the outputs (dopamine and melatonin) of these two oscillators.

MATERIALS AND METHODS

Animal maintenance

Juvenile green iguanas (*Iguana iguana*) (30 to 50 g) obtained from Glades Herpetological Supply (Fort Myers, FL, USA) were housed in plastic cages within a wooden enclosure on a light:dark (LD) 12:12 cycle (lights-on 0600 h, lights-off 1800 h). White light was provided by a fluorescent lamp (20 μ W) (Philips, Somerset, NJ, USA), and additional heat from a thermal pad maintained the cage temperature at 28 ± 2 °C. Animals were fed fresh green vegetables three times per week, with water supplied ad libitum.

Melatonin Extraction and Radioimmunoassay

Eyes from halothane-anesthetized animals were harvested in darkness using an infrared viewer (Find-R-Scope, FJW Optical Systems, Palatine, IL, USA), immediately frozen in individual Eppendorf tubes, and stored at -80 °C. Melatonin chloroform extraction and radioimmunoassay (RIA) were performed as described by Rollag and Niswender (1976); this assay has been validated for iguanas (Tosini and Menaker, 1996b). Eyes were homogenized in 200 μ L of phosphate buffer with 0.9% NaCl and 1% gelatin (phosphate-buffered saline gel) and sonicated using a microultrasonic cell disrupter (Kontes, Vineland, NJ, USA). Melatonin was extracted in 1 mL of chloroform and washed with 200 μ L of sodium-carbonate buffer (0.1 M, pH 10.25). Samples were spun and supernatant removed. Aliquots were dried under nitrogen and suspended in 0.5 mL of phosphate-buffered saline gel. RIA was performed using melatonin antibody (R1055) and I125-melatonin analog (Covanzel, Vienna, VA, USA).

Dopamine Quantification by High-Pressure Liquid Chromatography

Levels of dopamine and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured by high-pressure liquid chromatography (HPLC) (Beckman

System Gold®, San Ramon, CA, USA). Tissue was homogenized in 200 μ L of 80% 0.1 M perchloric acid/20% acetonitrile solution and the supernatant spin-filtered through a 0.2 μ m nylon filter (PGC Scientifics, Gaithersburg, MD, USA). Twenty microliters of supernatant were injected into a Microsorb® Short-One C18 reverse phase column (3 μ m, 2.8 $\times$ 10 cm) (Varian Associates, Walnut Creek, CA, USA). Mobile phase consisted of 75 mmol phosphate buffer with 25 mmol EDTA, 1.7 mmol 1-octanesulfonic acid, 0.01% triethylamine, and 7% acetonitrile (pH 3). Quantification was carried out with an ESA Coulochem detector (ESA Inc., Bedford, MA, USA) fitted with a 5020 guard cell set at +400 mV, a 5011 analytical cell with a glassy carbon screening electrode set at 75 mV, and a measuring electrode at +250 mV. All separations were performed at a flow rate of 1.0 mL/min. Peaks and relative concentrations were identified by comparison with known external standards.

ERGs

ERGs were obtained in constant darkness (DD) and in dim (5 μ W/cm²) LD cycles (dLD 12:12) as described previously (Miranda-Anaya et al., 2000). Light intensity was calibrated at the level of the head of the animals using a radiometer (model 350, Graseby Optronics, Orlando, FL, USA). A platinum-iridium loop electrode was positioned on the cornea of a urethane-anesthetized animal (2.5 g/kg) and covered with clear silicone. The reference electrode was placed subdermally in the front of the animal's head. Animals were held in a light-tight chamber with the temperature maintained at 28 °C using copper tubing and a circulating bath system (Lauda MGW, Westbury, NY, USA). A 250 ms light pulse (50 μ W/cm²) was given using a halogen lamp and delivered by a fiber optic located 3 mm from the cornea. Light irradiance was chosen to produce about 50% of maximal b-wave amplitude (Miranda-Anaya et al., 2000). ERGs were evoked every 15 min during at least two consecutive circadian cycles, amplified using a wide-band EEG-AC preamplifier (model 7P5 band-pass filter [3 to 1500 Hz], Grass, Quincy, MA) coupled to a DC driver (Grass model 7DA), and recorded on a polygraph (Grass model 79D). b-wave amplitude was defined as the voltage difference between the trough of the a-wave and the peak of the b-wave. In this study, the a-wave was not considered, since its amplitude was barely rhythmic and phase dependent effects could not be evaluated.

Protocol

Experiment 1: Melatonin and Dopamine Content in Control Conditions

Twenty-one iguanas were transferred from LD 12:12 to DD. Eyes from three animals were collected in the dark at projected circadian times (CTs) 2, 6, 10, 14, 17, 20, and 23 on the second day in DD, and kept at -80 °C until content determination. The period of the locomotor activity rhythm of the iguana in DD is very close to 24 h; therefore, in this study we assume the circadian time on the second day in DD as a projection of the LD conditions in which the animals were maintained (e.g., circadian time 12 was considered as the projected transition from light to dark). Melatonin content of one eye from each animal was determined by RIA, and dopamine content of the other eye was measured using HPLC as described above.

Experiment 2: Effects of Intraocular Injections of Quinpirole, SKF38393, Eticlopride, and Melatonin on ERG b-Wave Amplitude

Urethane-anesthetized iguanas were transferred from LD 12:12 to DD. During the second cycle in DD, and in dim red light, a total volume of 10 μ L of melatonin or dopamine receptor agonist or antagonist was injected through the sclera into the vitreous body of the eye using a Hamilton syringe fitted with a 26-gauge needle. Experimental animals received 5, 50, or 500 μ mol doses of either the selective D2-receptor agonist quinpirole hydrochloride (in 1% 0.1 N HCl) or melatonin (in 1% methyl alcohol); or a 100 μ mol dose of either the D2-receptor antagonist eticlopride or the D1-receptor agonist SKF38393 hydrobromide (in 1% 0.1 N HCl) (Sigma, St. Louis, MO, USA). Controls were injected with 10 μ L of vehicle (either 1% 0.1 N HCl, or 1% methyl alcohol). Changes in ERG b-wave amplitude induced by these agents were measured from animals held in DD with only the test light required to evoke the ERG. Injections were given near the middle of the projected subjective night (MSN) (2300 to 0100 h, EST) or near the middle of the projected subjective day (MSD) (1100 to 1400 h), when the lowest and highest amplitudes of ERG b-wave occur. For each agent, five ERGs taken at 1-min intervals before injection of the agent were used as a reference. After the injection, ERGs were obtained every minute for 15 min, and then every 15 min for at least 12 hours. Injections of

eticlopride were only given during the projected early subjective day (0900 h) to observe its effect on the presence of the diurnal peak of the b-wave amplitude.

Experiment 3: ERG Circadian Rhythm in Dopamine-Depleted Retinas

After 3 consecutive days of ERG recording in DD from intact animals ($n = 6$), intraperitoneal urethane anesthesia was reinforced with a second dose of urethane (0.8 g/kg), enabling us to work with fully anesthetized animals; injections of 6-hydroxy-dopamine (6-OHDA) (50 μ mol in 20 μ L) were then given in the vitreous body of both eyes. The anesthetized animals were held in LD 12:12 (lights-on 0700-1900 h) for 3 days after the injection, when a second dose of 6-OHDA was given. Four days after the second injection, anesthesia was again reinforced and ERGs were obtained for 3 more days in DD. During treatment with 6-OHDA, animals were administered a glucose solution through a feeding tube inserted into the esophagus.

Experiment 4: ERG Circadian Rhythm and Dopamine Content in Optic Nerve-Sectioned Eyes

Animals were anesthetized with urethane; additional anesthesia (10% procaine in saline) was injected into the palate. An incision was made in the palate, and the soft tissue was retracted with forceps until the optic nerve could be seen. One optic nerve was cut using a spring scissors, and the wound was packed with Gelfoam. Sham operations were done in the same way, except the optic nerve was not cut. ERG records were initiated immediately after surgery. In one group of animals ($n = 7$), ERGs were recorded from both eyes simultaneously, the eye with intact optic nerve serving as a control. In a second group ($n = 7$), only one eye was recorded from before and after surgery, thus serving as its own control. At the end of each record, animals were euthanized with an overdose of urethane and the optic nerve was visually inspected to ensure it had been completely severed. ERGs were obtained in either DD or dim LD. A third group of iguanas was used to evaluate daily changes in dopamine content of optic nerve-sectioned (ON-X) eyes in DD ($n = 9$), using HPLC as described in experiment 1. For these measurements, animals with one optic nerve sectioned were transferred from LD to DD, and both eyes were collected in darkness during the MSD ($n = 5$) and

in the MSN ($n = 4$) of the second cycle in DD. The intact eye of each animal was used as the control for the ON-X eye.

Data Analysis

In each experiment, circadian rhythmicity of ERG b-wave amplitude was calculated at a 95% level of confidence using a fast Fourier transform–nonlinear least squares estimation method developed by Martin Straume (National Science Foundation, Center for Biological Timing, University of Virginia; see Plautz, 1997). Circadian variations in content of melatonin and dopamine (experiment 1) were evaluated using analysis of variance; the effect of intraocular injections (experiment 2) was evaluated using the Kruskal-Wallis nonparametric test and contrasted with the Dunnett test by means of Sigma-Stat software (Jandel Scientific, San Rafael, CA, USA). The Mann-Whitney test was used to compare dopamine content at two different time points (experiment 4). A p value < 0.05 was considered significant. Because the iguanas' free-running periods are very close to 24 h, subjective time was considered as the projection of the previous LD conditions in which the animals were kept before the experiments. Thus, subjective day corresponds to the previous interval between lights-on and lights-off.

RESULTS

Experiment 1: Melatonin and Dopamine Content in DD Conditions

Both melatonin and dopamine content in the eye show robust circadian rhythms ($p < 0.01$). Figure 1A shows that the average ($\pm$ SEM) melatonin content of eyes taken during the beginning of the subjective night (CTs 14 and 17, black bars) is more than five times the level observed during the subjective day (gray bars). The highest levels of melatonin ($407.08 \pm \text{SEM } 9.13$ pg/eye) occurred at CT 14, and the lowest levels of melatonin (75.57 ± 6.55 pg/eye) at CT 2. The peak levels of dopamine and melatonin are nearly 180 degrees out of phase with each other. The highest levels of dopamine occurred at CT 23 (2143.7 ± 61.6 pg/eye) (Fig. 1B) and the lowest levels (1450 ± 2.4 pg/eye) at CT 17. Dopamine metabolites DOPAC and HVA also showed a significant circadian pattern ($p < 0.05$) (Fig. 1C).

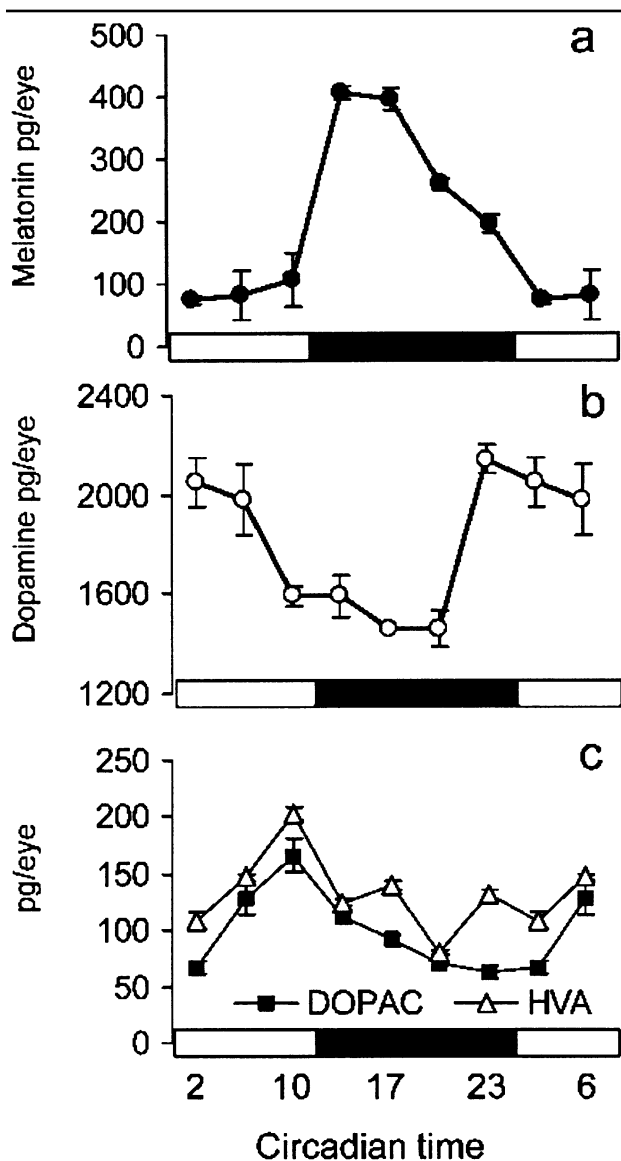


Figure 1. Temporal profiles of (A) melatonin and (B) dopamine content ($\pm$ SEM; $n = 21$) in the eyes of intact iguanas held in constant darkness. Melatonin levels are high during the subjective night (black bars), and dopamine levels are high during the subjective day (gray bars). (C) Profiles of dopamine metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA); both peak at the time when dopamine synthesis drops most precipitously.

Experiment 2a: Effects of Dopamine Receptor Agonist and Antagonist

Fifty micromolars of quinpirole acutely increased the b-wave amplitude when injected intraocularly at MSN but not at MSD. Figure 2A shows the expected peak of the b-wave amplitude near MSD of the first

cycle in DD and the increase in b-wave amplitude produced by intraocular injection of quinpirole in the middle of the second subjective night. Figure 2B shows the average b-wave amplitude ($\pm$ SEM) before and after injection of quinpirole or vehicle in the MSN. B-wave amplitudes of quinpirole-injected animals were significantly higher than in vehicle-injected animals 7 min after the injection, and continued rising to about 30% above controls. When injected at MSD, the effect of the same dose of quinpirole did not differ from the effect of vehicle (Fig. 2C). Average amplitudes observed 10 to 15 min after injection of vehicle or 5, 50, or 500 μ mol of quinpirole during MSD and MSN are shown in Figure 2D. When injected at MSD, only 500 μ mol of quinpirole showed a clear effect compared to vehicle ($p < 0.05$, Kruskal Wallis test), whereas at MSN injection of both 50 and 500 μ mol produced significant effects ($p < 0.05$). Ten microliters of the D2 receptor antagonist eticlopride (100 μ mol) injected at the beginning of the subjective day acutely suppressed b-wave amplitude, which then slowly recovered over the next 12 h; however, the normal peak in b-wave amplitude was not seen in the cycle after the injection in the three animals tested (Fig. 3A). b-wave amplitude of animals injected with the D1-receptor agonist SKF38393 (100 μ mol) at either MSN (Fig. 3B) or MSD (data not shown) did not differ significantly from that of vehicle-injected controls. SKF38393 caused a slight rise in the amplitude of the b-wave between 3 and 6 minutes after the injection, but it returned to the reference values quickly (Fig. 3C).

Experiment 2b: Melatonin Decreases the b-Wave Amplitude during the Subjective Day

Figure 4A shows the decrease in b-wave amplitude caused by injection of 50 μ mol of melatonin during MSD. Figures 4B and 4C show the effects on b-wave amplitude of the same dose of melatonin administered during MSN and MSD, respectively. Melatonin injected during MSN caused only a slight drop in amplitude (Fig. 4B, closed circles) and did not differ significantly from vehicle-injected controls (open circles), whereas injection of melatonin at MSD (closed circles) caused a rapid drop in b-wave amplitude that stabilized after 5 min at about 40% of the vehicle-injected controls (Fig. 4C). Figure 4D shows the dose-response curve for the effect of melatonin on b-wave amplitude. Significant differences ($p < 0.05$, Kruskal-

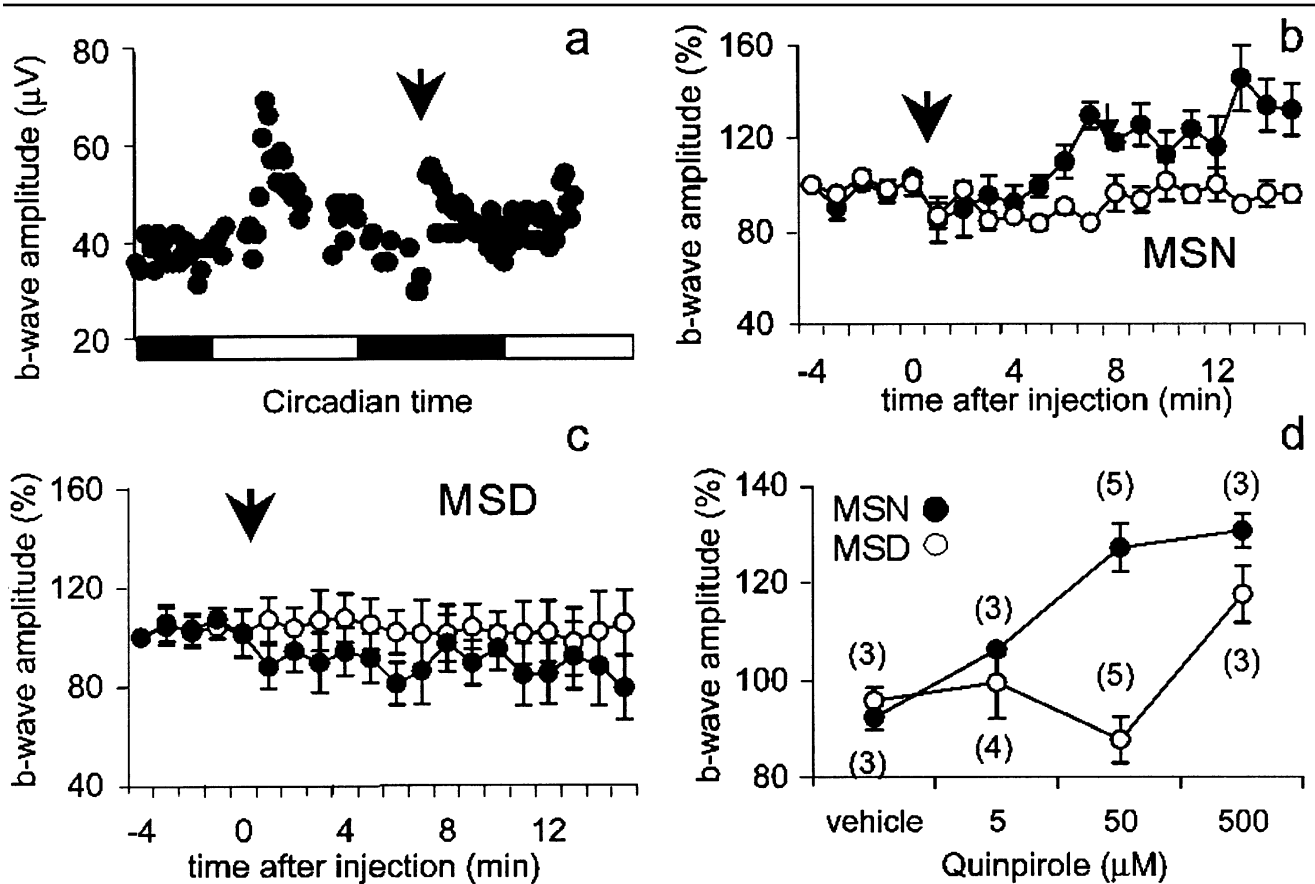


Figure 2. Intraocular injections of quinpirole increase electroretinogram b-wave amplitude. (A) A typical record in which b-wave amplitude is high in the middle of the subjective day (MSD). At the following middle of the subjective night (MSN), a 50-μmol injection of quinpirole (arrow) produces a clear increase in b-wave amplitude similar to that normally observed during MSD. Dashed bars represent the subjective day, and MD on the time axis marks midday. (B, C) The time course of changes in b-wave amplitude produced by injection (arrow) of 50 μmol of quinpirole (solid circles, $n = 5$) or vehicle (open circles, $n = 3$) at MSN (B) and MSD (C). The dose response relationship for quinpirole injections at MSN (solid circles) and MSD (open circles) (percentage average $\pm$ SEM) are shown in (D). The number in parentheses represents the total number of animals used at each point.

Wallis test) were observed using concentrations of 50 and 500 μmol of melatonin compared with the vehicle at MSD, but there were no significant effects at MSN.

Experiment 3: Dopamine Depletion Abolishes the Rhythm of b-Wave Amplitude

In this experiment each animal was used as its own control. Figure 5A shows the circadian profile of b-wave amplitude for three cycles in an intact animal. After treatment with 6-OHDA, the ERG of the same animal appeared normal but the b-wave amplitude was not rhythmic and it remained at the level observed during MSN (Fig. 5B). Similar results were observed in five of six animals treated with 6-OHDA; one animal showed a dramatic change in period (con-

trol $\tau = 24$, 6-OHDA $\tau = 18.5$ and 64 h) (data not shown).

Experiment 4: Optic Nerve Sectioning Abolishes the Circadian Rhythm of Dopamine Content and b-Wave Amplitude

Figure 6A shows the rhythm of b-wave amplitude in an intact animal in DD. At the beginning of the fourth cycle, the optic nerve was sectioned (arrow). After surgery, the b-wave was arrhythmic during the next three cycles in DD, and its amplitude stabilized at levels below those previously observed during the subjective night. Circadian rhythmicity of b-wave amplitude was abolished in all four animals subjected to ON-X.

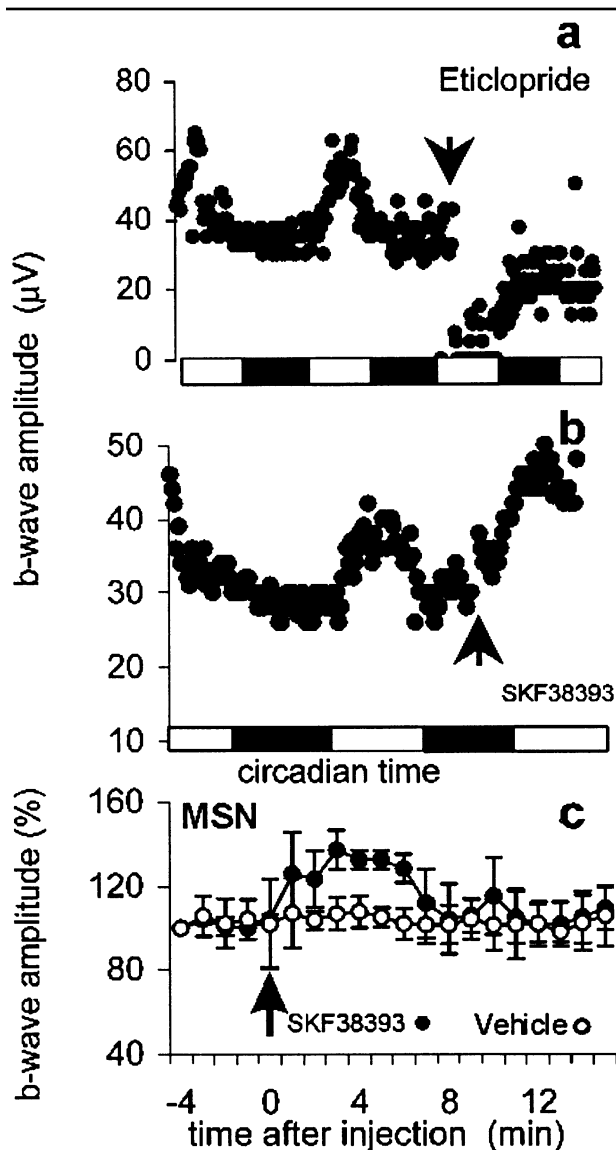


Figure 3. Intraocular injections of eticlopride decrease b-wave amplitude (A). Injections given at the beginning of the subjective day abolish the subsequent peak. A slight effect of the D1 agonist SKF38393 on the b-wave amplitude is shown in (B) and (C). An acute increase in b-wave amplitude was seen after the injection of SKF38393 (arrow), but its effect lasts only about 6 min ($n = 3$). MSN = middle of the subjective night.

When both eyes were recorded simultaneously, four of seven animals showed a complete lack of circadian rhythmicity of b-wave amplitude in the ON-X eye compared to the intact control eye (Fig. 6B). In the other three records, the ERG from ON-X eyes was rhythmic on the first day after nerve section but damped completely by the second cycle (data not shown). Sham-operated animals ($n = 2$) showed a clear rhythm in both eyes (Fig. 6C). Dopamine content at

MSD and MSN in control and ON-X eyes held in DD is compared in Figure 7. There was a five-fold difference between dopamine levels at MSD (1406.56 ± 292.36 pg/eye) and MSN (258.2 ± 78.27 pg/eye) in control eyes but no significant difference between MSD and MSN levels in ON-X eyes ($p > 0.05$, Mann-Whitney test).

Although b-wave amplitude was not rhythmic in DD following ON-X, it was rhythmic in the presence of a light cycle. Figure 8A shows the b-wave amplitude recorded from an ON-X eye during the first 3 days in DD after surgery, followed by 3 days of dLD cycles. A daily rhythm in b-wave amplitude can be seen during the last 2 days of the record, with higher amplitude during the light. In Figures 8B and 8C, b-wave amplitude was recorded from the intact eye (8B) and an ON-X eye (8C) simultaneously. A daily rhythm can be observed in the ON-X eye, with higher amplitude during the light ($n = 3$). Furthermore, dopamine content of ON-X eyes during the light portion of an LD cycle does not differ from that of controls ($p > 0.05$, Mann-Whitney test) (data not shown), suggesting that dopamine levels are rhythmic in ON-X eyes in LD, although not in DD (Fig. 7).

DISCUSSION

Circadian Rhythms of Melatonin and Dopamine Content in the Eye of Iguana In Vivo

Melatonin and dopamine are known to have opposite effects on several aspects of retinal physiology, and factors that influence dopamine release also influence the synthesis of melatonin (Besharse et al., 1988). Indeed, circadian rhythms in the retina may be reinforced by a feedback loop between melatonin and dopamine synthesis (Adachi et al., 1999; Grace et al., 1999). The circadian oscillator that regulates melatonin synthesis is located in the iguana retina (Tosini and Menaker, 1998), but although dopamine is rhythmic in the eyes of intact iguanas, this rhythm does not persist in vitro. Furthermore, in vivo it is abolished by optic nerve section, indicating that regulation of retinal dopamine synthesis is primarily neural and that the oscillator regulating it is in the brain. Therefore, it seems that circadian rhythmicity in several aspects of retinal function may depend on the interaction of at least two spatially separate circadian oscillators.

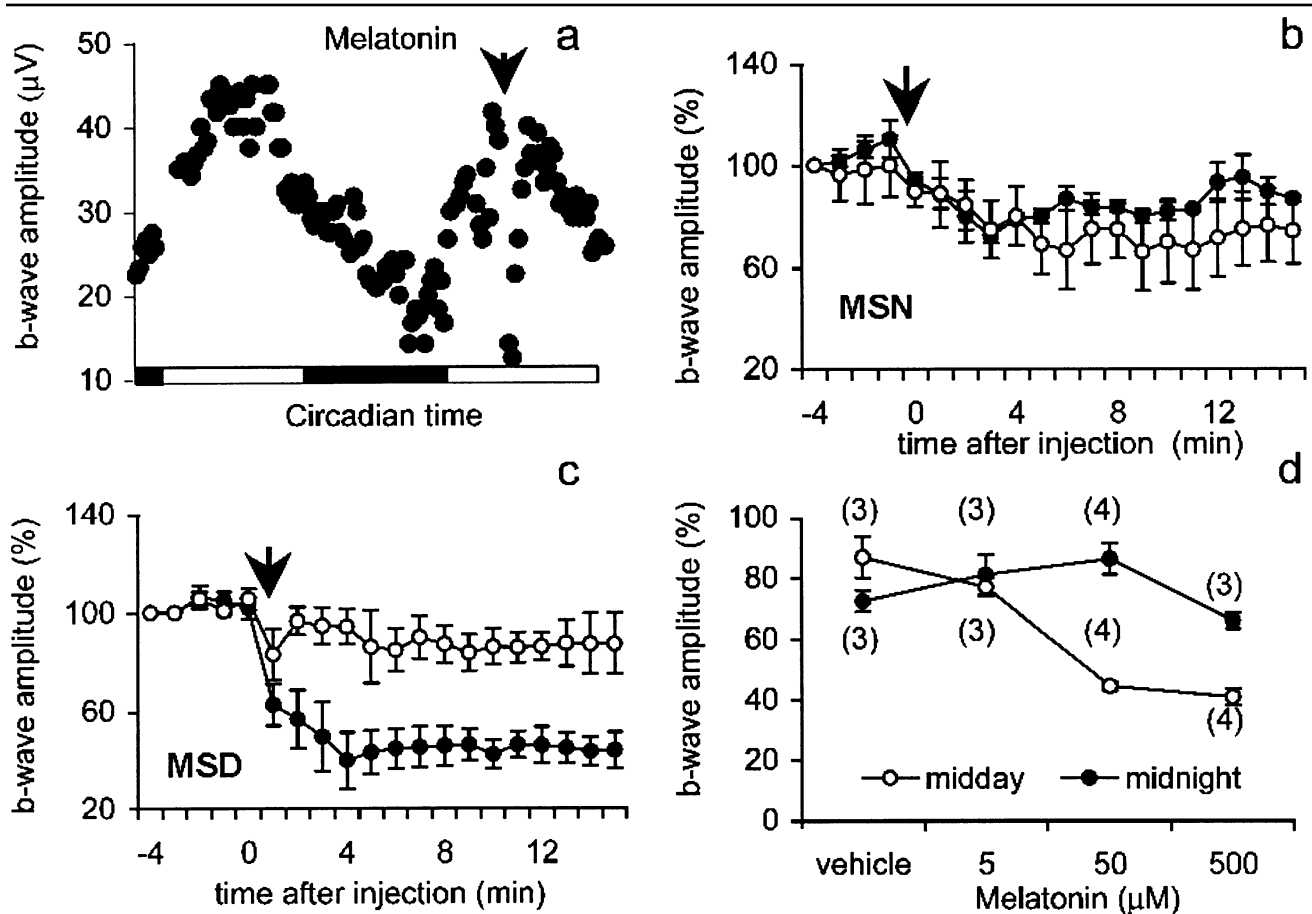


Figure 4. Effect of intraocular melatonin injections on b-wave amplitude. (A) A typical circadian rhythm occurs during the first cycle in constant darkness; at the middle of the subjective day (MSD) of the second cycle, an injection of 50 μmol of melatonin decreases the b-wave amplitude from peak to subjective night levels. The effects of injections of melatonin (solid circles) and vehicle (open circles) during the first 15 min following the injections are shown in (B) and (C), respectively. The time of injection is indicated by the arrow. The dose response curves for injections of melatonin given at MSD (open circles) and the middle of the subjective night (MSN) (solid circles).

Dopamine Modulates the ERG Circadian Rhythm through D2 Receptors

Dopamine has been shown to participate in light adaptation mechanisms in retinas of several species, and enhancement of ERG b-wave after intraocular injection of dopamine has been observed in light-adapted retinas of chicken (Fujikado, 1994). The results of our experiments with quinpirole, eticlopride, and 6-OHDA indicate that the circadian rhythm of ERG b-wave amplitude is certainly modulated and perhaps driven by dopamine acting primarily through D2 receptors.

The ERG b-wave is the result of electrical activity of the optic nerve bipolar cells expressed through currents in the Müller cells, as a result of increased extracellular potassium produced by depolarization of retinal neurons (Stockton and Slaughter, 1989;

Dowling, 1987). Optic nerve bipolar cells receive inputs from amacrine cells, where most dopamine is produced (Nguyen-Legros, 1996; Engbretson and Battelle, 1987; Bartell et al., 2000). Dopamine D2 agonist quinpirole increases rod-cone photoreceptor coupling in the frog (Krizaj et al., 1998) and modulates glutamate-gated ionic currents in bipolar cells of the tiger salamander (Maguire and Werblin, 1994). In reptiles, junctions between horizontal cells and photoreceptors have been described (for a review, see Peterson, 1992). Although it is interesting to speculate on the function of dopamine in the circadian regulation of visual sensitivity in iguana, we should do so with caution. Our results show that in the green iguana, the peak of b-wave amplitude occurs during the subjective day, when dopamine levels are high. Therefore, dopamine may be involved in the retinal mechanisms that increase light sensitivity when it is

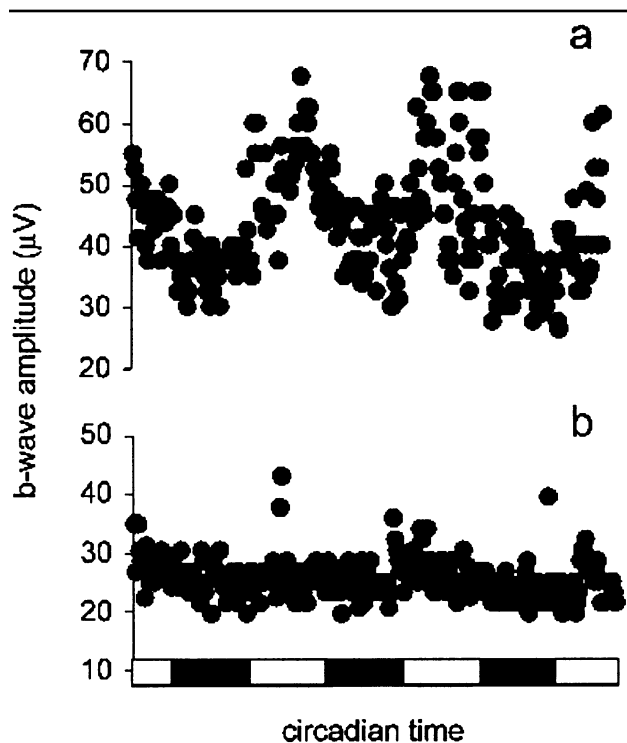


Figure 5. The circadian rhythm of an electroretinogram is lost after depletion of dopamine in the retina. After the record shown in (A) was obtained, the animal was treated with two intravitreal injections of 6-hydroxy-dopamine 3 days apart. When the same eye was retested 1 week after the first injection, no rhythm could be observed (B).

present for extended periods, possibly by modulating photoreceptor coupling via the horizontal cells.

The daytime peak in b-wave amplitude in the iguana is correlated with the amount of dopamine present in the retina; the absence of a rhythm in b-wave amplitude in dopamine-depleted retinas is consistent with this observation and has also been reported in fish (Li and Dowling, 2000) and in Japanese quail (Manglapus et al., 1999). In quail, there is a circadian rhythm of b-wave amplitude that, in contrast to that in the iguana, peaks at night. The effects of quinpirole and eticlopride in iguana retina were opposite to those observed in quail: b-wave amplitude in quail is reduced by quinpirole injected during the subjective night and increased by eticlopride injected during the subjective day. Because the iguana retina comprises primarily cones (Meneghini and Hamasaki, 1967), a circadian rhythm in rod-cone dominance is unlikely to occur as it does in quail. Because the phases of the rhythms of melatonin and dopamine synthesis are similar in quail and iguana (Underwood and Siopes, 1984; Manglapus et al., 1999), whereas the phase of the rhythm of b-wave amplitude and the

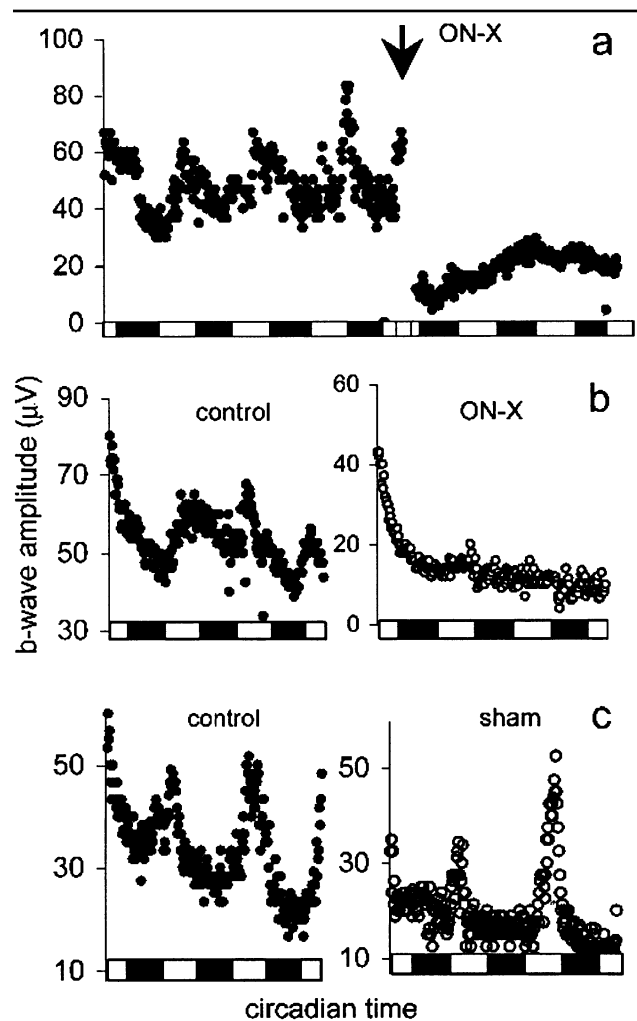


Figure 6. (A) Circadian rhythm in b-wave amplitude of an intact animal during four cycles before optic nerve-sectioned (ON-X, arrow); after the optic nerve is sectioned (in the light), the rhythm is lost. (B) Simultaneous records in constant darkness of b-wave amplitude from the control eye with a statistically significant circadian rhythm (solid circles) and the arrhythmic b-wave amplitude in the ON-X eye (open circles). (C) Simultaneous records from a control eye (solid circles) and a sham-operated eye (open circles); both are clearly rhythmic.

effect of quinpirole and eticlopride are opposite, it seems likely that dopamine exerts its effects on the ERGs of the two species by different mechanisms.

Melatonin Is Involved in the Modulation of the ERG Circadian Rhythm

Retinal melatonin is produced primarily during the subjective night both in vitro and in vivo when b-wave amplitude is at its lowest. During the subjective day when b-wave amplitude is high, intraocular melatonin injection significantly reduces it, which

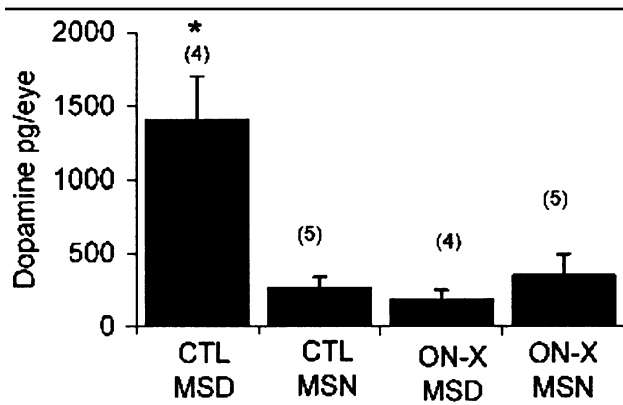


Figure 7. Control eyes (CTL) have significantly more dopamine in the middle of the subjective day (MSD) than in the middle of the subjective night (MSN), and significantly more than optic nerve-sectioned (ON-X) eyes at either time. The asterisk indicates that the CTL MSD value is significantly different ($p < 0.05$) from that of all the other groups; there are no other significant differences.

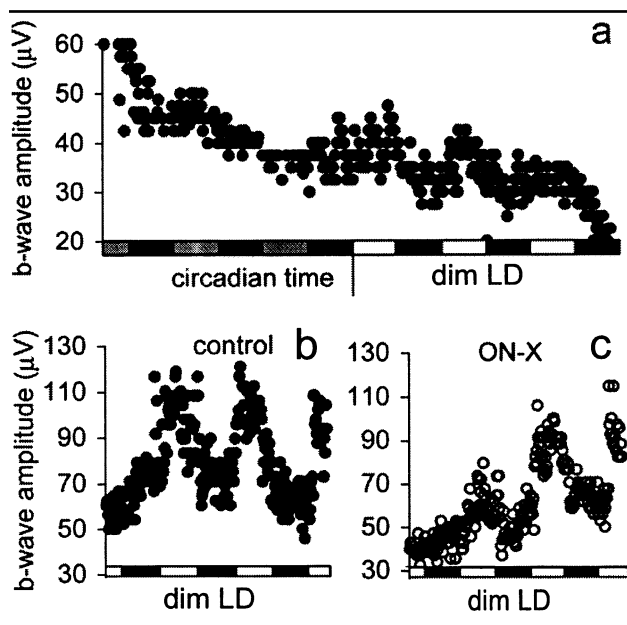


Figure 8. (A) b-wave amplitude in an optic nerve-sectioned (ON-X) eye is arrhythmic in constant darkness but is rhythmic in the presence of a dim light:dark (dLD) cycle. (B, C) Simultaneous recordings from a control (solid circles) and an ON-X eye (open circles) in dLD.

suggests that melatonin may be involved in its regulation. Melatonin injected during the subjective night does not affect b-wave amplitude, perhaps because the retina is already responding maximally to endogenous melatonin.

Melatonin is known to be involved in the circadian regulation of several aspects of retinal physiology (for a review, see Cahill and Besharse, 1995; Cahill and

Hasegawa, 1997; Pozdeyev et al., 2000). Intramuscular injections of melatonin affect b-wave amplitude in fowl in a phase-dependent manner (Lu et al., 1995). Continuously administered melatonin abolishes the circadian rhythm of b-wave amplitude in chicks (McGoogan and Cassone, 1999). We have shown previously that pinealectomy affects the amplitude as well as the period of the circadian rhythm of b-wave amplitude in iguana, but rhythmicity itself does not depend on pineal melatonin (Miranda-Anaya et al., 2000). The effect of exogenous intraocular melatonin reported here suggests that melatonin, rhythmically produced by the retina, may decrease the amplitude of the b-wave in vivo, perhaps by inhibiting the synthesis of dopamine as it does in the eyes of pigeons (Adachi et al., 1999) and rabbits (Dubocovich, 1983). These observations support the concept of reciprocal inhibition between retinal melatonin and dopamine. Further support for this idea comes from in vitro studies, which show that the D2-receptor agonist quinpirole inhibits the synthesis of melatonin in the cultured retinas of *Xenopus* (Cahill and Besharse, 1991), hamsters (Tosini and Dirden, 2000), and iguanas (unpublished data).

Optic Nerve Sectioning Abolishes the ERG Circadian Rhythm

The retina sends information to the brain, but the optic nerve also contains fibers that send information from the brain to the retina, forming a feedback loop that may control events in the retina, including its sensitivity to light. The retinal dopaminergic system in fish is partially controlled in this way by neural projections from the retinopetal nervus terminalis cells in the olfactory bulb (for a review, see Ball et al., 1989). The abolition of circadian rhythms of b-wave amplitude and dopamine content by optic nerve section suggests that in the iguana, these rhythms are also regulated via afferents to the retina through the optic nerve. The daily rhythms in b-wave amplitude in ON-X eyes exposed to dim LD cycles may be the result of dopamine synthesis in the retina that is directly induced by light, as can be seen in ON-X eyes.

The circadian rhythm of b-wave amplitude observed during the first cycle in some iguanas with one optic nerve severed may indicate that a secondary oscillator in the retina is driving the rhythm, but damps quickly without neural input from a primary oscillator in the brain. This retinal oscillator could be the one that regulates the rhythmic synthesis of

melatonin. Although melatonin rhythmicity persists in cultured retinas and, presumably, after ON-X, persistence of the circadian rhythm in b-wave amplitude requires a circadian rhythm of retinal dopamine. The participation of other neural or humoral inputs in the modulation of the ERG circadian rhythm cannot be excluded by our data. Based on the anatomy of two other lizards, *Ctenosaura pectinata* and *Cyclura carinata* (Underwood, 1970), it is possible that our ON-X procedure could damage nearby nerves, or the blood supply from the palatine and/or temporal arteries. However, because the circadian rhythm of b-wave amplitude persists in sham-operated eyes and can be driven by light in ON-X eyes, we do not think that the effects of ON-X are due to nonspecific damage.

ERG Circadian Rhythm Regulation by Negative Feedback of Dopamine-Melatonin

In Figure 9, we propose a model of the mechanism that generates the circadian rhythm of ERG b-wave amplitude in the iguana retina. An oscillator in the brain regulates retinal dopamine rhythmicity through centrifugal pathways, whereas a retinal oscillator controls rhythmic melatonin synthesis. During the subjective day, dopamine increases b-wave amplitude directly via D2 receptors and by inhibiting melatonin synthesis. During the subjective night, melatonin depresses b-wave amplitude and may, at the same time, inhibit the synthesis of dopamine. Not shown in the model but also consistent with our data is the possibility that light may directly increase b-wave amplitude by direct stimulation of retinal dopamine synthesis.

Diurnal versus Nocturnal Circadian Rhythm of ERG in Vertebrates

Although the reciprocal inhibition of retinal dopamine and melatonin appears to be generally consistent among vertebrate species, the phase of the peak in b-wave amplitude is not. The phase differences observed have been attributed to differences in habitat (Rojas et al., 1999; Fowlkes et al., 1987), rod-cone dominance (Manglapus et al., 1998), or experimental protocol (Wu et al., 2000; McGoogan et al., 2000). In our experiments, the b-wave always peaked in the day or subjective day even though we varied the reference

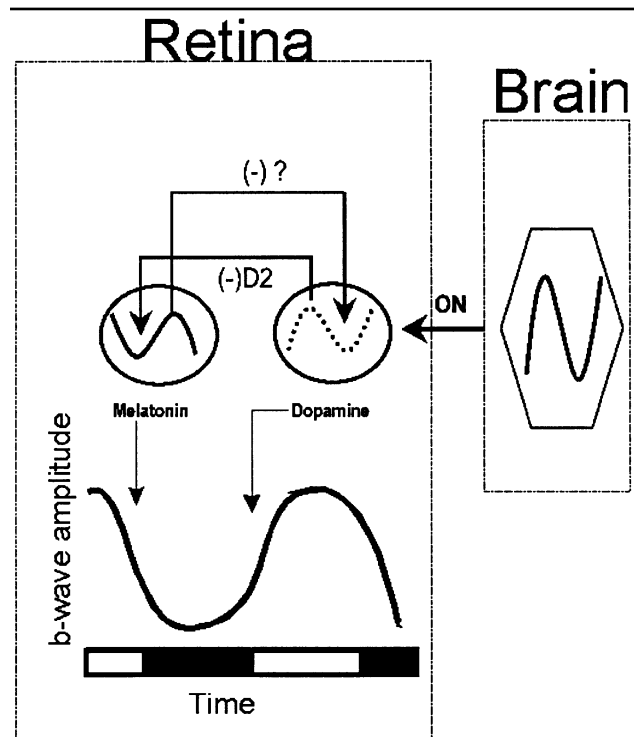


Figure 9. Model of the mechanism that may generate the circadian rhythm of electroretinogram b-wave amplitude in the iguana. An oscillator in the brain regulates or reinforces the circadian rhythm of dopamine synthesis in the retina via the optic nerve. High levels of dopamine during the subjective day increase b-wave amplitude. The retina contains an oscillator that controls the synthesis of melatonin (dotted line); in addition, melatonin synthesis is inhibited by dopamine (and light if present). Melatonin, synthesized during the subjective night, directly depresses b-wave amplitude and may inhibit dopamine synthesis. During the subjective day, dopamine may repress melatonin synthesis and increase the amplitude of the b-wave.

electrode placements and other aspects of the methodology, and we therefore propose, as a working hypothesis, that the phase of the b-wave peak depends on selective changes in the responsiveness of two (or more) populations of cones. We are testing this idea by examining circadian changes in spectral sensitivity. Of course this, and other aspects of retinal function, is likely to have evolved to maximize adaptation to a particular ecological niche.

ACKNOWLEDGMENTS

The work was supported by a grant (56647) from the National Institute of Mental Health. We thank Bahie Rassekh for assistance with electroretinograms and surgeries, Kelly Coffey for tissue collection, and Wilson McIvor for assistance in high-pressure liquid

chromatography. Mark Rollag generously donated the antibody for melatonin, and Carla Green and Naomi Ihara provided much helpful discussion.

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