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Cellular analysis of the *Bulla* ocular circadian pacemaker system

II. Neurophysiological basis of circadian rhythmicity

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Summary. We have used chronic, or long-term, intracellular recording combined with simultaneous extracellular recording of optic nerve activity to examine the neurophysiological basis of circadian rhythmicity in the *Bulla* eye. We report that:

1. Continuous intracellular recordings from R-type photoreceptors were maintained for up to 28 h. These recordings reveal that in constant conditions R-type cells do not exhibit rhythms in membrane potential which correlate with the circadian rhythm in compound action potential frequency expressed by the eye.

2. Continuous intracellular recordings from basal retinal neurons were maintained for up to 74 h. These recordings reveal that in constant conditions basal retinal neurons exhibit clear circadian rhythms in membrane potential and action potential frequency which are synchronized with the circadian rhythm in compound action potential frequency. Action potentials in individual basal retinal neurons correlate one-for-one with the compound action potentials in the optic nerve over the entire circadian cycle. The basal retinal neurons depolarize during the active phase of the compound action potential rhythm (projected day), relative to their membrane potential during the inactive phase of the rhythm (projected night).

3. The phase relationship between the rhythm in basal retinal neuron membrane potential and action potential frequency is such that the rise in membrane potential from its most hyperpolarized point precedes, or is synchronous with, the increase in action potential frequency observed near projected dawn. This suggests that the membrane potential rhythm drives the circadian rhythm in impulse frequency.

4. The quantitative relationship between basal retinal neuron membrane potential and action potential frequency is not linear, and varies predictably with circadian phase. Following the interval of peak impulse frequency the rate of impulse production declines more rapidly than does the membrane potential. Also, the impulse frequency at a given membrane potential is lower during the falling phase of the circadian cycle than during the rising phase.

5. In conclusion, we find that the basal retinal neurons are at minimum a pacemaker output pathway, and are likely the circadian pacemaker itself. We find no role for the R-type photoreceptor in the generation of circadian rhythmicity by the *Bulla* eye.

Abbreviation: BRN basal retinal neuron

Introduction

The eye of the marine mollusc *Bulla gouldiana*, like the eyes of several other opisthobranch molluscs, contains a circadian pacemaker which expresses a circadian rhythm in the frequency of spontaneous optic nerve impulses when isolated and maintained in constant conditions (*Aplysia:* Jacklet, 1969; *Bulla:* Block and Friesen 1981; *Bursatella:* Block and Roberts 1981; *Haemonea:* McMahon and Block 1982; *Navanax:* Eskin and Harcombe 1977). Of these molluscan retinae, the *Bulla* eye has proved particularly advantageous for electrophysiological analysis of its circadian pacemaker. The preceding paper has described the organization of the *Bulla* retina. The present study was undertaken to investigate the neurophysiological basis of circadian rhythmicity in the *Bulla* eye by the use of chronic, or long-term, intracellular recording techniques.

The *Bulla* retina is composed of two principal cell layers (Block and Wallace 1982; Jacklet and Colquhoun 1983). There is a photoreceptor layer

consisting of approximately 1000 elongate photoreceptor cells which surround the large central lens, and, at the base of the retina, there are the basal retinal neurons (BRNs), of which there are approximately 100 in each *Bulla* eye. Tissue reduction experiments have demonstrated that a competent circadian pacemaker system: photic entrainment pathway, circadian oscillator, and pacemaker output pathway, resides in retinal base fragments which contain a few BRNs, but do not contain cells from the photoreceptor layer (Block and Wallace 1982; Block and McMahon 1984). These experiments demonstrate that cells in the photoreceptor layer are not critical for the generation of circadian rhythmicity by the *Bulla* eye. However, cells in this layer may be rhythmic, and while we have found no evidence of direct connections between individual R-type photoreceptors and BRNs, a weak electrical connection cannot be excluded (Block et al. 1984). We have also proposed that the H-type cells of the photoreceptor layer make inhibitory connections with the BRNs (Block et al. 1984). Thus, cells in this layer could have a modulatory effect on the compound action potential rhythm even though they are not essential for generation of the rhythm.

While surgical reduction procedures are useful for identifying the cells in the *Bulla* retina which are critical for expression of the circadian rhythm, the technique lacks utility for studying the cellular events underlying fundamental pacemaker properties, such as rhythm generation and entrainment. In this study we used long-term intracellular recording, a procedure which monitors the state of individual cells in the pacemaker, in order to examine the cellular-level neurophysiological events involved in the circadian pacemaker mechanism of the *Bulla* eye. Intracellular recordings of short duration have already provided evidence that BRNs play an important role in the *Bulla* ocular circadian pacemaker system. These recordings demonstrate that BRNs are contributing units to the compound action potentials which express the circadian rhythm, and that manipulation of BRN membrane potential by current injection alters the compound action potential frequency (Block et al. 1984).

Since both tissue reduction and electrophysiological evidence establish basal retinal neurons as candidate circadian pacemaker neurons we have developed the techniques for long-term intracellular recording from these neurons, and have begun to examine their circadian physiology. We have also extended earlier studies of cells in the photoreceptor layer using long-term intracellular recording (Block and Wallace 1982).

The results of the present study indicate that individual basal retinal neurons exhibit circadian rhythms in action potential frequency and membrane potential which are synchronized with the observed circadian rhythm in optic nerve compound action potential frequency. Thus the BRNs are certainly a pacemaker output pathway and are likely the circadian pacemaker itself. In contrast, cells from the photoreceptor layer fail to exhibit rhythmic variations in membrane potential which correlate with the compound action potential rhythm. We find no role for the R-type photoreceptors in the generation of circadian rhythmicity by the *Bulla* eye.

Preliminary reports of some of these experiments have been published (Block and Wallace 1982; McMahon and Block 1983).

Methods

Bulla gouldiana were obtained from West Coast marine suppliers and maintained as previously described (Block et al. 1984). Eye removals were performed on *Bulla* immobilized with injections of 5–7 ml of isotonic $MgCl_2$, and cooled on ice. In order to prepare the isolated eyes for recording, the lens was removed by the method described previously (Block et al. 1984), and the connective tissue was removed from one side of the eye with iridectomy scissors. The eye was then pinned out in a Sylgard-coated petri dish containing sterile artificial seawater, and its optic nerve pulled into a suction electrode for extracellular recording. The temperature of the bath was maintained at 15 °C by a Lauda K2/R recirculating cooler. Following impalement with conventional glass capillary microelectrodes (50–100 MOhm) filled with 3 mol/l KCl, the eyes were maintained in continual darkness. Intracellularly recorded signals were amplified with a WPI M707 electrometer, and recorded on a Grass polygraph together with the extracellular signals from the optic nerve.

Extracellular optic nerve recordings were analyzed by counting and plotting the number of compound action potentials occurring per half-hour interval. The time at the end of each half-hour 'bin' was taken as the reference point for the interval. Intracellularly recorded membrane potential is reported as the average membrane potential for each half-hour interval during the recording. The average membrane potential for each half-hour interval was calculated by determining the membrane potential at 5 min intervals from the intracellular record, and then averaging the 6 measurements. During intervals of impulse activity by BRNs the average membrane potential was determined by ascertaining the membrane potential at the midpoint between impulses closest to the 5 min marks. The time reference point was again taken as the time at the end of the interval.

Results

Long-term intracellular recording from photoreceptors

In order to investigate the possibility that cells in the photoreceptor layer exhibit circadian rhythmi-

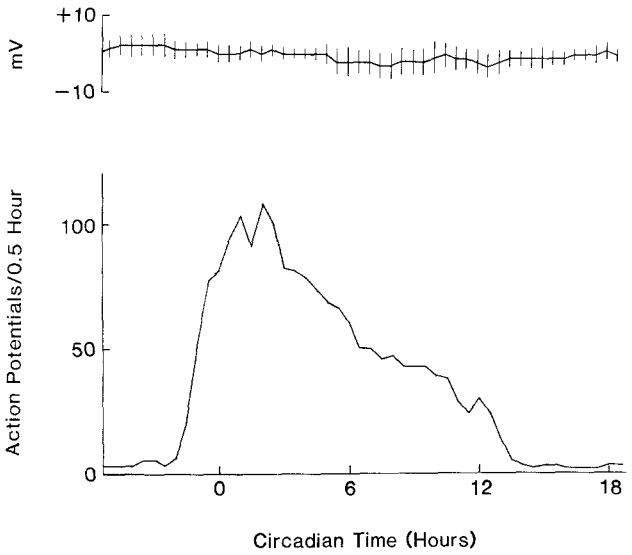

Fig. 1. Simultaneous intracellular recordings from photoreceptors and extracellular recordings from the optic nerve. Compound action potential frequency and average membrane potential were determined for each half-hour interval in seven long-term recordings (7–28 h). These recordings were then aligned in circadian time (previous dawn = circadian time 0) and averaged. Upper panel shows relative membrane potential. Vertical bars: standard deviations. All standard deviations overlap the 24 h mean membrane potential. Lower panel shows the average compound action potential frequency. There is no correlation between photoreceptor membrane potential and compound action potential frequency (correlation coefficient = −0.09)

city, we monitored the resting membrane potential of photoreceptors by long-term intracellular recording while simultaneously monitoring compound action potential activity in the optic nerve. The most common cell type found in the photoreceptor layer are R-type cells. These photoreceptors depolarize in response to light and do not exhibit somatic action potentials (Block et al. 1984). Stable, continuous intracellular recordings from R-type photoreceptor cells were maintained for up to 28 h. We obtained seven long-term records ranging in duration from 7 to 28 h. Two of the recordings were maintained for a full circadian cycle, or longer. All seven records were aligned in circadian time and averaged to produce Fig. 1. There is no detectable circadian rhythmicity in membrane potential in these photoreceptor recordings, and no correlation between fluctuations in photoreceptor membrane potential and compound action potential frequency (correlation coefficient = −0.09). The mean amplitude of the range in resting membrane potential in all seven photoreceptor records is 5 mV. One recording showed a large fluctuation in resting membrane potential (16 mV) which, again, did not correlate with the compound action potential frequency. The other six recordings showed smaller fluctuations in resting membrane potential, with the ranges of membrane potential varying from 3 to 6 mV. Because of the difficulty in maintaining impalements of H-type cells (Block et al. 1984) long-term intracellular recording from these cells was not feasible.

Long-term intracellular recording from basal retinal neurons

In contrast to photoreceptor recordings, long-term intracellular recordings from basal retinal neurons revealed that individual BRNs exhibit pronounced circadian rhythms in action potential frequency and membrane potential which are synchronized with the observed circadian rhythm in compound action potential frequency. Figure 2 shows a series of 12 min sample traces taken from a continuous BRN recording. This particular eye was entrained to an LD 12:12 light cycle with dawn at 0900 (EST), and following impalement was maintained in continual darkness. The circadian cycle of BRN activity has the expected phase relationship to the previous light cycle for the *Bulla* ocular circadian rhythm (Block and McMahon 1984). The BRN was largely inactive during the night, and its membrane potential was below the −60 mV reference line on the record (0000, 0300). By 0600 it depolarized, initiating impulse activity a few hours before projected dawn, and it was most depolarized near its peak in action potential frequency (0900). The BRN repolarized as action potential frequency fell during the projected day (1200, 1500, 1800), and finally became fully repolarized and inactive again during the projected night (2100, 2400).

We have been able to maintain continuous intracellular recordings from individual basal retinal neurons in the *Bulla* eye for up to 74 h. Figure 3 illustrates the circadian rhythms in membrane potential and action potential frequency exhibited by a BRN during 60 h of a 74 h recording. Both parameters exhibit two pronounced circadian cycles and a third cycle of reduced amplitude. The circadian rhythms in action potential frequency recorded from this BRN, and shown in the bottom part of the figure, are precisely synchronized with the circadian rhythm in compound action potential frequency recorded simultaneously from the optic nerve. That is, the one-for-one correlation between BRN action potentials and compound action potentials in the optic nerve is preserved throughout the entire recording period. The upper panel of the figure shows the circadian rhythms in membrane potential exhibited by the BRN during the recording period. There is a clear correlation be-

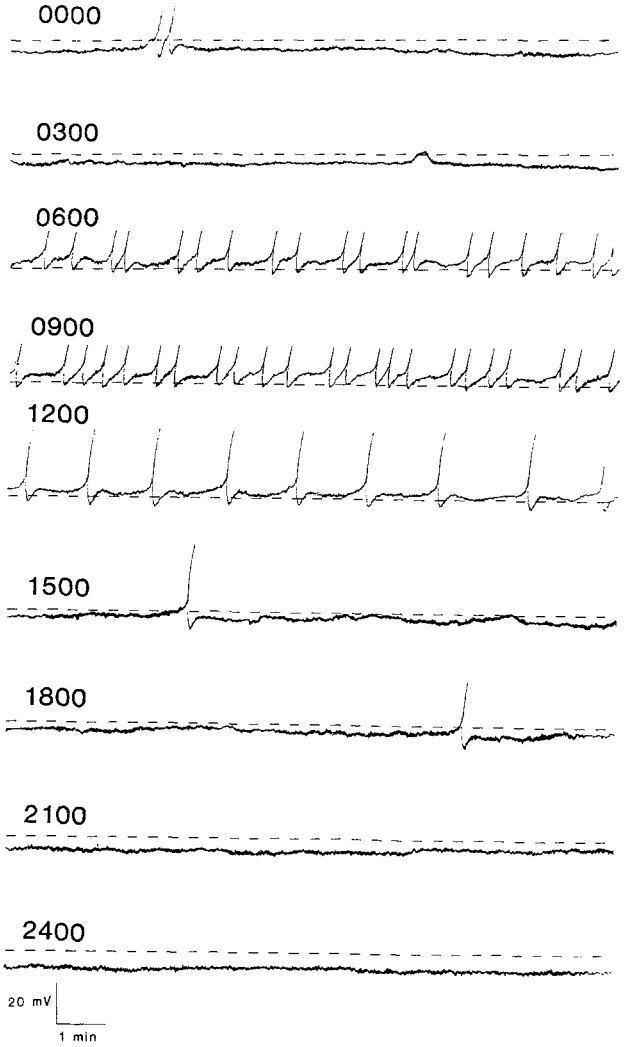

Fig. 2. Long-term intracellular recording from a basal retinal neuron illustrating circadian rhythms in membrane potential and action potential frequency. Each line represents a 12 min segment from a continuous intracellular BRN recording. The time (EST) of each segment is shown on the left. This eye was taken from an animal entrained to an LD 12:12 light cycle with previous dawn at 0900. Dashed line in each trace represents the −60 mV level and serves to illustrate the circadian rhythm in membrane potential exhibited by the BRN. Action potential height is attenuated due to clipping

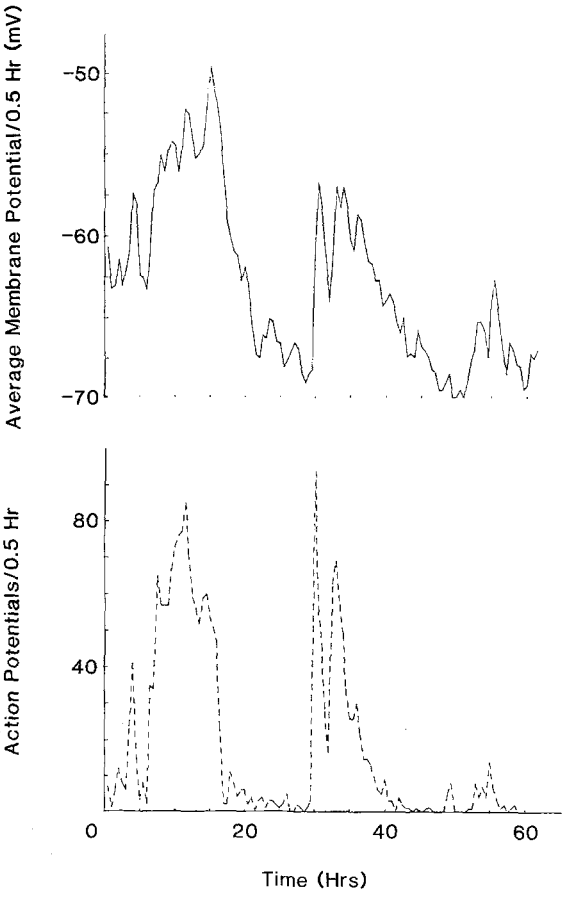

Fig. 3. Circadian rhythms in membrane potential and action potential frequency exhibited by a basal retinal neuron during a continuous intracellular recording. Upper panel shows the average membrane potential of BRN for each half-hour interval during the 60 h of recording. The average membrane potential moves slowly toward more negative values during recording period. Lower panel shows action potential frequency expressed by the BRN. Rhythms in membrane potential and action potential frequency are synchronized with each other and with the circadian rhythm in compound action potential frequency expressed by the eye

tween the membrane potential of the BRN and impulse frequency. The amplitude of the membrane potential rhythm in the BRN as measured from the trough to the following peak is 14 mV on the first circadian cycle, 12 mV on the second circadian cycle, and is 7 mV on the damped cycle. While the 74 h recording is the longest we have obtained to date, we have obtained 5 other recordings of significant duration (3–20 h). In the other recording in which the full excursion of the membrane potential rhythm was recorded the amplitude of the rhythm was 13 mV trough to peak. The same correlation between BRN membrane potential and action potential frequency is present in all of these records.

The temporal relationship between the circadian rhythms in membrane potential and impulse frequency is illustrated in Fig. 4. The rise in basal retinal neuron membrane potential from its most hyperpolarized level either precedes the increase in impulse activity by the BRN near projected dawn by at least one half-hour interval, or it occurs within the same half-hour interval, and is not found to follow the rise in action potential frequency. In the case shown in Fig. 4 the depolarization of the BRN precedes the increase in impulse activity by about 1.5 h. After the interval of peak impulse frequency it was often the case that im-

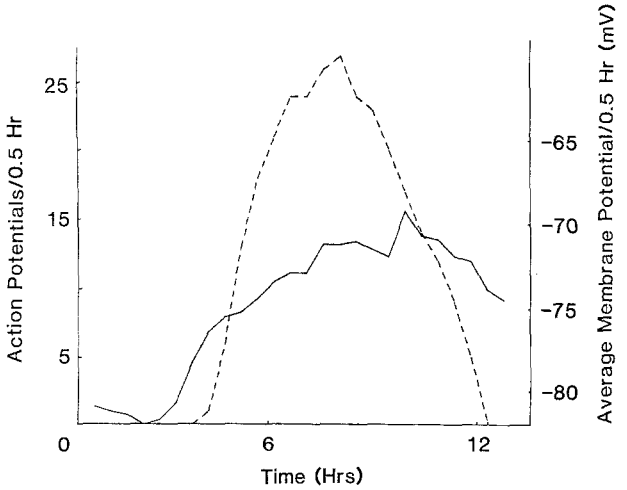

Fig. 4. Temporal relation of rhythms in basal retinal neuron membrane potential and action potential frequency. The rise in BRN membrane potential (solid line) from its most hyperpolarized point, precedes initiation of impulse activity in the BRN (dashed line). Following the peak in impulse frequency there is an interval when the action potential frequency falls more rapidly than the membrane potential does

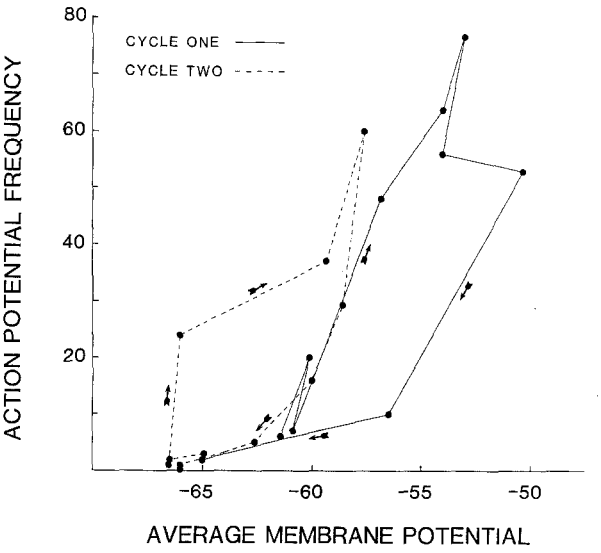

Fig. 5. Quantitative relationship of basal retinal neuron membrane potential and action potential frequency during the circadian cycle. Average membrane potential (horizontal axis) and action potential frequency (vertical axis) were determined for each 2 h interval during the first two circadian cycles of the recording shown in Fig. 3, and the data points connected in chronological order. Solid line connects points from the first cycle, and dashed line connects points from the second cycle. Arrows: chronological sequence of data points. Time proceeds clockwise on both cycles. The second cycle is displaced to the left of the first, indicating the average membrane potential is more negative during the second cycle. This reflects the trend of the average membrane potential shown in Fig. 3

pulse frequency fell while membrane potential remained stable or even depolarized slightly (right half of Fig. 4).

The time-dependent variation in the quantitative relationship between membrane potential and action potential frequency is illustrated in Fig. 5. Here, the membrane potential and action potential frequency were averaged for each two hour interval during the first two cycles of the record shown in Fig. 3. To generate the figure these data points were connected in chronological order. Time progresses in a clockwise direction on both cycles. The salient feature is that the quantitative relationship between BRN membrane potential and action potential frequency is different during the rising and falling phases of the circadian cycle. During the rising phase, when impulse frequency was increasing, the rate of impulse production was higher at a given membrane potential than during the falling phase, when impulse frequency was decreasing. Interestingly, the lines tracing the rising and falling phases are nearly parallel. Thus, the change in impulse frequency engendered by a given change in membrane potential is almost the same, but the line has been displaced downward (fewer action potentials/time at a given membrane potential) on the falling phase. This displacement occurs during the interval following peak impulse frequency when the rate of action potential production declines more rapidly than does membrane potential. These trends were exhibited in all BRN recordings in which some parts of both the rising and falling phases were recorded ($N=3$).

Discussion

The role of the photoreceptors

Our results from long-term intracellular recordings from R-type photoreceptors indicate that these cells do not exhibit rhythmic variations in membrane potential which correlate with the circadian rhythm recorded in the optic nerve. This confirms an earlier finding from this laboratory (Block and Wallace 1982). However, since our recording location in the photoreceptor soma appears to be electrically distant from the spike initiating zone of these cells (Block et al. 1984) we cannot rigorously exclude the possibility of a small circadian pacemaker potential occurring at this site. Nevertheless, we consider the participation of R-type cells in the compound action potential rhythm to be unlikely. In addition to their lack of overt rhythmicity, R-type cells do not drive the compound action potential response to light (McMahon and Block 1982; Block and McMahon 1983), nor do they have a demonstrable connection with the BRNs (Block et al. 1984). Thus, the membrane potential of the R-type photoreceptors appears to have no role in the generation of circadian rhyth-

micity by the *Bulla* eye. Strumwasser and coworkers (1979) have reached a similar conclusion for the *Aplysia* eye based on long-term recordings from isolated photoreceptors maintained in cell culture.

Circadian rhythmicity in basal retinal neurons

Our long-term intracellular recordings from individual basal retinal neurons reveal that these neurons exhibit circadian rhythms in membrane potential and action potential frequency which underlie the observed circadian rhythm in compound action potential frequency. Action potentials in these neurons occur one-for-one with the compound action potentials in the optic nerve over the entire circadian cycle. Thus, individual BRNs contribute to all phases of the circadian cycle, the population of BRNs acting synchronously to express the compound action potential rhythm. This synchrony is not surprising since *Bulla* BRNs are electrically coupled (Block et al. 1984).

The membrane potential rhythm expressed by the BRNs is such that during the active phase of the compound action potential rhythm the basal retinal neurons depolarize relative to their membrane potential during the inactive phase of the rhythm. The rise in BRN membrane potential precedes or is synchronous with, the increase in compound action potential frequency near projected dawn. In addition, manipulation of BRN membrane potential by current injection induces changes in impulse frequency in the optic nerve (Block et al. 1984). Injecting depolarizing current increases compound action potential frequency, while injecting hyperpolarizing current inhibits compound action potentials. These observations suggest that the membrane potential rhythm in the BRNs drives the circadian rhythm in action potential frequency in these neurons, thus producing the circadian rhythm in optic nerve impulse frequency.

The relationship between basal retinal neuron membrane potential and action potential frequency is not simple and linear, but changes during the course of the circadian cycle. As Fig. 5 illustrates there is an interval following peak impulse frequency when the rate of impulse production falls more rapidly than does membrane potential. This non-linearity may be due to a change in the threshold of the BRN spike generating mechanism. Or, since the loci of rhythm generation and action potential production have been shown to be anatomically distinct (Block and McMahon 1984), it is possible that these regions do not remain isopotential at elevated spike frequencies. An alternative explanation is that the action potential generating mechanism is not a passive element driven by the membrane potential rhythm, but is part of the circadian pacemaker mechanism and interacts with the membrane potential rhythm to generate the 24 h periodicity. We consider this possibility to be unlikely since in the related opisthobranch *Aplysia* complete blockage of all action potentials with 6 h pulses of TTX-Hi-Mg-Lo-Ca solutions does not phase shift the ocular circadian rhythm (Eskin 1977).

Basal retinal neurons: Candidate circadian pacemaker neurons

Evidence from both tissue reduction and electrophysiological experiments leads us to propose that the basal retinal neurons are circadian pacemaker neurons. We have formed a 'working hypothesis' on the cellular organization of the circadian pacemaker in the *Bulla* retina based on our experimental results to date. For a detailed description of our model of the *Bulla* ocular circadian pacemaker system, and a complete review of the evidence in support of it, the reader is referred to the following paper (Block and McMahon 1984). Briefly, in the proposed model each *Bulla* BRN contains a circadian pacemaker which expresses a circadian rhythm in membrane potential. This membrane potential rhythm modulates the activity of an anatomically distinct impulse generating area of the BRN neurite. The BRNs are electrically coupled at the level of these spike generating areas, and thus give rise to compound action potentials in the optic nerve which express a circadian rhythm.

Because the basal retinal neurons are tightly electrically coupled, it is not certain to what extent the rhythms in membrane potential and action potential frequency recorded from any one BRN soma represent intrinsic rhythmicity, and to what degree they may reflect rhythms generated in other cells in the BRN population. We have considered three possible mechanisms which could give rise to the circadian rhythms in BRN membrane potential and action potential frequency. First, each BRN is a competent circadian oscillator and generates its own circadian rhythm. Here, electrical coupling would serve only to promote synchrony in the population of circadian pacemaker neurons. Second, only a subpopulation of the BRNs are circadian pacemaker neurons, and they drive the rhythms in membrane potential and action potential frequency in the other BRNs through the electrical junctions. Third, a separate and distinct cell type at the base of the retina generates the circadian rhythm, and imposes rhythmicity on the BRNs. These 'driver' cells might be neurons connected to the BRNs, or perhaps glia, which could rhythmically alter the extracellular environment surrounding the BRNs. Potential 'driver' cells would have to be electrically or ionically coupled

to the BRNs since the circadian rhythm is not abolished in Hi-Mg Lo-Ca seawater solutions (McMahon and Block, manuscript in preparation). These solutions have been shown to block chemical transmission in *Bulla* and *Aplysia* eyes (Block and McMahon 1983, 1984). Our model incorporates the first alternative, that each basal retinal neuron is a competent circadian oscillator because it is the simplest explanation which accounts for our results. We have found the BRN population to be morphologically, electrophysiologically, and functionally homogeneous so explanations involving subpopulations, or separate cell types as the locus of circadian rhythm generation are unattractive (see Block and McMahon 1984).

While the evidence suggesting basal retinal neurons are circadian pacemaker neurons is considerable, it is still circumstantial. Unambiguous identification of BRNs as the circadian pacemaker in the *Bulla* eye requires further experimentation, and it is our intention that the proposed model yield specific and testable hypotheses on this question. One such prediction is that individual BRNs should continue to exhibit circadian rhythmicity in isolation from the rest of cells in the retina. Long-term recording from individual BRNs maintained in dispersed cell culture would be the most convincing test of this prediction.

The proposed model also focuses attention on the role of basal retinal neuron membrane potential in the *Bulla* ocular circadian system. The circadian rhythm in membrane potential in these neurons is hypothesized to drive the compound action potential rhythm, and thus represents an output of the circadian pacemaker. Experiments using ionic and pharmacological agents have demonstrated that membrane potential changes also play a role in the entrainment of the ocular circadian pacemaker of *Aplysia* (Eskin 1977, 1982). One prediction of our hypothesis that the BRNs are the circadian pacemaker in the *Bulla* eye is that these cells would be affected by entrainment stimuli. This prediction is borne out by the observation that light pulses depolarize *Bulla* BRNs (Block and Friesen 1981; Block et al. 1984), and produce a phase response curve similar to the *Aplysia* phase response curve to light pulses (Block and McMahon 1984). In addition, preliminary results in our laboratory indicate that the phase-shifting agent serotonin, which in *Aplysia* produces a phase response curve essentially opposite to that of light (Corrent et al. 1982), generates a prolonged hyperpolarization in *Bulla* BRNs. Thus BRN membrane potential may play a role both in the entrainment and the overt expression of the circadian pacemaker in the *Bulla* eye. The question arises as to whether membrane potential is a fundamental component of the circadian oscillator mechanism, or merely a passive element. We believe the role of membrane potential in the *Bulla* ocular circadian pacemaker system is a critical issue which when experimentally addressed will yield fundamental information on the mechanisms of entrainment and generation of circadian rhythmicity at the cellular level.

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