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Cellular analysis of the *Bulla* ocular circadian pacemaker system

III. Localization of the circadian pacemaker

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Summary. The isolated *Bulla* eye expresses a circadian rhythm in optic nerve impulse frequency. In an effort to determine the anatomical location of the circadian pacemaking system within the retina we surgically reduced the eye. We report that:

1. The approximately 1000 large photoreceptors which form a cell layer immediately surrounding the lens, are not required for the expression of a circadian rhythm. Eyes which are surgically reduced so that only the basal retinal neuron population remains, continue to express a circadian rhythm indistinguishable in period to intact eyes.

2. The photoreceptor layer is also not required for light-induced phase shifts of the ocular rhythm. Retinal fragments containing only basal retinal neurons can be phase advanced or delayed by 6 h light pulses provided at the appropriate circadian phase.

3. Of the approximately 100 basal retinal neurons in the *Bulla* eye, only a small proportion are required for the expression of a circadian rhythm in optic nerve frequency. Ocular fragments with as few as 6 basal retinal neuron somata remain rhythmic, and exhibit a free-running period indistinguishable from intact eyes.

4. Intact basal retinal somata are required for the expression of a circadian rhythm in optic nerve impulse frequency. Retinal fragments consisting of an optic nerve with a small amount of neuropil region produce spontaneous action potentials without evidence for a circadian modulation.

5. An explicit model for the organization of the circadian pacemaker system in the *Bulla* retina is proposed.

Abbreviation: BRN basal retinal neuron

Introduction

A major goal of circadian rhythms research on metazoans is to identify and characterize cells or cell circuits which give rise to circadian oscillations. The opisthobranch eye has been an important model system for studying circadian oscillators since the retinae of several opisthobranchs including *Aplysia* (Jacklet 1969), *Bursatella* (Block and Roberts 1981), *Haemonea* (McMahon and Block 1982) and *Navanax* (Eskin and Harcombe 1977) express precise circadian rhythms in vitro. Of these molluscan preparations, the ocular circadian pacemakers of *Aplysia* and *Bulla* have been the most intensively studied. Efforts to locate the circadian pacemaking system contained within the *Aplysia* retina have revealed that a retinal fragment containing approximately 100 photoreceptor cells and other types of neurons, supports a circadian periodicity (Strumwasser 1973). The proximity of different cell populations to one another within the *Aplysia* retina has made it difficult to surgically isolate and identify a particular cell type as the locus of the circadian pacemaker. By selectively exposing different regions of the retina to ionizing radiation it has been found however, that cells in the posterior portion of the eye are more likely the site of the pacemaking system (Woolum and Strumwasser 1981). In addition, it has been reported that isolated photoreceptors maintained in culture do not exhibit circadian rhythms in resting membrane potential or light response, also implicating cells outside the photoreceptor layer as the site of the circadian pacemaker (Strumwasser et al. 1979).

The *Bulla* retina offers several advantages over *Aplysia* for cellular studies of circadian oscillators.

It contains approximately one fifth the number of cells found in the *Aplysia* retina (Block and Wallace 1982; Jacklet and Colquhoun 1983), and there is better spatial separation between morphologically distinct cell populations. This allows for a more systematic reduction of retinal tissue and thus offers the possibility of identifying a particular cell type as a circadian pacemaker locus. The morphology of *Bulla* retinal cells has also allowed for long-term intracellular recording from both photoreceptors, and neurons at the base of the retina (Block and Wallace 1982; McMahon et al. 1984). These studies reveal that neurons at the base of the retina express circadian periodicities in membrane potential and impulse frequency while cells in the photoreceptor layer (R-type receptors) fail to exhibit rhythmic fluctuations in membrane potential. These results suggest that the circadian pacemaker property resides among cells at the base of the retina.

In the present study, we have attempted to identify the location of the circadian pacemaking system within the eye by surgically reducing the normal complement of retinal cells. Our results indicate that only neurons at the base of the retina are required for the expression of a circadian rhythm. We find that the circadian rhythm persists in retinal fragments containing as few as six basal retinal neurons, suggesting that individual neurons are competent circadian pacemakers. Furthermore, surgically reduced retinas containing only basal retinal neurons remain photosensitive and can be phase-shifted by light pulses. Finally, combining information we have gathered from tissue reduction experiments with previous electrophysiological studies (Block et al. 1984; McMahon et al. 1984), we provide an explicit model for the organization of the circadian pacemaking system in the *Bulla* eye. A brief report of some of these experiments has recently been published (Block and Wallace 1982).

Methods

Bulla gouldiana were obtained from West Coast marine suppliers and maintained in our laboratory as previously described (Block et al. 1984). All animals were exposed to light cycles generated by 2–40 W 'Cool White' fluorescent tubes (Light: Dark, 12:12, 1500 lux) for at least 1 week prior to experimentation.

The eyes were surgically reduced as follows: each eye, and its attached optic nerve, was first removed from the animal and the optic nerve pulled into a U shaped suction electrode so that the lens was facing upward. A small slit was then made in the 'cornea' with iridectomy scissors and the lens was gently prodded out of the connective tissue capsule which surrounds the eye. Following lens removal, the eye was transected across the base with iridectomy scissors, removing most of the retinal tissue. Typically only 50–100 μm of retinal tissue surrounding the head of the optic nerve remained after this surgical procedure. The tissue reduction operation lasted approximately 15 min, after which time the surgically reduced eye and a control eye from the same animal were transferred to a 150 ml petri dish containing fresh, buffered artificial seawater (30 mmol/l HEPES with 100 units/ml penicillin, 100 mcg/ml streptomycin). The eyes were then placed in an incubator (16 °C) and optic nerve activity recorded extracellularly for at least 48 h in continual darkness.

Following recording, retinal fragments were fixed in a 2.0% glutaraldehyde, 1.5% formaldehyde solution (mixed in HEPES buffered artificial seawater) for 12–24 h at room temperature, dehydrated and embedded in methacrylate. Thick serial sections (1–2 μm) were made of each fragment. Sections were stained with acid fuchsin and toluidine blue and then observed with a compound microscope. The number of retinal somata remaining after surgery was estimated by counting the number of nuclei visible in the serial sections.

Analysis of optic nerve recordings consisted of counting and plotting the number of compound action potentials in each half-hour interval. The half-hour interval closest to the half maximum impulse frequency (midrise) was taken as the phase reference point for each rhythm. The free-running period was calculated by measuring the interval between the midrise points of the first and second cycles.

The determination of whether surgically reduced eyes were rhythmic was accomplished by visual inspection of the records of spontaneous impulse frequency. We considered eyes rhythmic if they continued to exhibit well defined peaks in impulse frequency at circadian intervals (20–30 h). In practice we found that surgically reduced eyes continued to exhibit robust circadian rhythms or appeared entirely non-periodic. The short duration of in vitro ocular recordings (60–72 h) precluded the use of time-series analysis such as periodograms to evaluate possible rhythmicity (Enright 1981).

Results

Optic nerve rhythm

The *Bulla* eye produces spontaneous compound action potentials when maintained in continual darkness. These compound impulses, which are recorded from the optic nerve, are generated by the synchronous firing of a population of neurons located at the base of the retina (Block et al. 1984). The eye expresses a circadian rhythm in the frequency of these impulses both in vitro (Block and Friesen 1981) and in vivo (Block and Davenport 1982). Similar to *Aplysia,* the free-running rhythm is robust, with most eyes expressing 5–7 circadian cycles of spontaneous impulse activity in vitro when maintained in buffered seawater and continual darkness (Fig. 1). The mean free-running period of the isolated but intact *Bulla* eye is 23.7 h (± 0.5 h SD, $N=10$) measured midrise to midrise during the first two cycles and the mean impulse frequency is 64 impulses per half-hour (± 15.6 h SD, $N=23$).

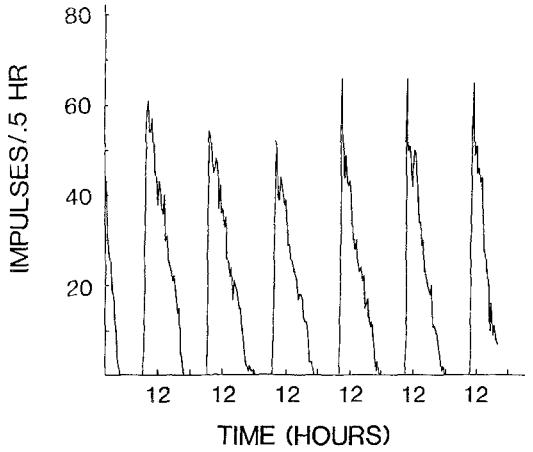

Fig. 1. Ocular circadian rhythm in *Bulla* eye. Optic nerve activity from an isolated eye was recorded in continuous darkness at 16 °C. Abscissa is clock time in hours (EST), ordinate shows the number of compound action potentials. The previous light cycle was L:D 12:12 with dawn at 09:00

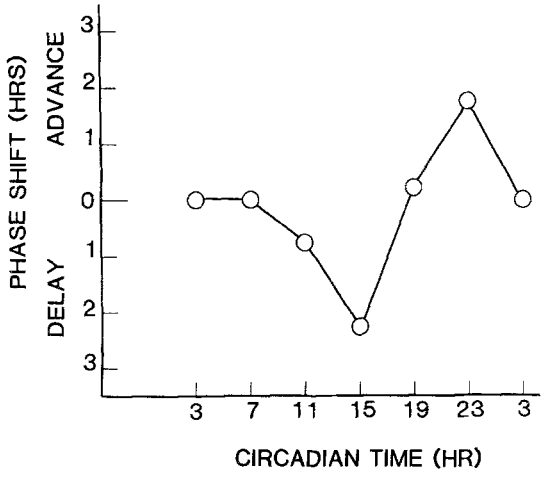

Fig. 2. Phase response curve of the *Bulla* ocular pacemaker to 6 h light pulses. Graph shows phase shifts produced in the rhythm as a function of the Circadian Time of exposure of the eyes to a light pulse. Pulses were given during the first day in isolation. Light was supplied by a light pipe illuminated by a high intensity fiber optic supply. Light intensity approximately 5000 lux. Data are plotted with respect to the midpoint of the light pulse. Each point represents the average of two eyes, except at hour 23 which is the average of 7 eyes and at hour 15 which is the average of 4 eyes. Circadian Time 0 equals light onset, and Circadian Time 12 equals light offset of the previous light cycle

The *Bulla* eye can be entrained by light cycles in vivo. When exposed to light cycles (L:D = 12:12), half maximum impulse frequency occurs 0.9 h (1.5 h SD, $N=26$) before projected dawn. The phase response curve for 6 h light pulses presented to an isolated eye, in vitro, is shown in Fig. 2. The eye exhibits phase dependent responses

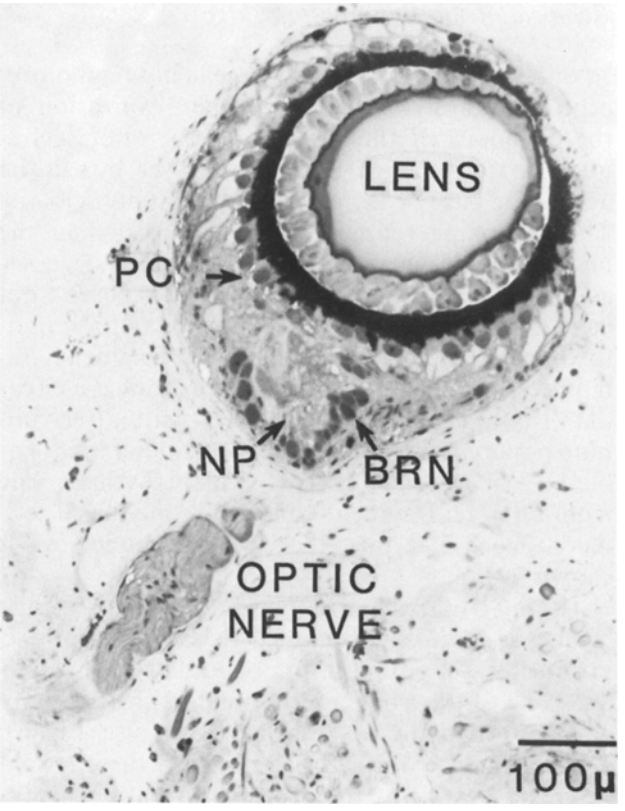

Fig. 3. Thick section of *Bulla* retina. From the lens inward, the stratified retina consists of the distal segments of photoreceptors, a pigmented region and a somatic layer containing the nuclei of the photoreceptors (*PC*). Near the base of the retina is a group of approximately 100 basal retinal neurons (*BRNs*) which surround a dense neuropil (*NP*) region

to light pulses typical of most circadian pacemakers. Phase delays occur in the early projected night followed by phase advances in the late projected night. Overall, the *Bulla* phase response curve to light pulses is similar to that obtained for *Aplysia,* although the *Aplysia* phase response curve is of higher amplitude (Corrent et al. 1982).

Ocular morphology. There are two principal cell layers within the *Bulla* retina (Fig. 3). Surrounding a central lens is a layer of photoreceptor cells with clearly defined distal segments containing microvilli. Interdigitating with these cells are pigmented support cells. There are approximately 1000 large photoreceptors in this layer (Block et al. 1984) as well as smaller photoreceptors, visible in thin sections (Jacklet and Colquhoun 1983). A second cell population, located near the retinal base, is composed of approximately 100 neurons which lack obvious photoreceptive specializations. Due to the location of these cells we have referred to them as basal retinal neurons (Block et al. 1984).

Removal of the photoreceptor layer

In order to determine whether cells in the photoreceptor layer are required for the expression of the circadian rhythm in optic nerve impulse frequency, we removed this retinal layer by cutting across the base of the eye with iridectomy scissors. The remaining retinal fragment consisted of the basal retinal neuron population (BRNs), associated neuropil and attached optic nerve, but lacked the somatic and distal portions of the photoreceptors (Fig. 4). These retinal fragments remained spontaneously active and exhibited a circadian rhythm in impulse frequency with a free-running period indistinguishable from intact eyes (Table 1). An example of two ocular rhythms, one from a retinal fragment containing only basal retinal neurons and the other from an intact eye is shown in Fig. 5.

Retinal fragments in which the photoreceptor layer was removed continued to respond to light, producing compound action potentials but exhibiting none of the smaller amplitude impulse activity which normally occurs during a light pulse (Fig. 6). The persistence of a light response in these retinal fragments confirms the presence of light sensitive neurons at the retinal base and is consistent with our findings using local illumination techniques (Block and McMahon 1983; Block et al. 1984). Effective photic entrainment pathways persist in retinal fragments containing only basal retinal neurons. Six hour light pulses delivered to retinal fragments at Circadian Time 2000 engendered a $+1.1$ h phase advance (± 0.8 h SD, $N=7$) while a six hour pulse at Circadian Time 1200 caused a mean phase delay of -1.3 h (± 0.7 h SD, $N=5$) when compared to the phase of unshifted control eyes. An example of a light-induced phase delay in a retinal fragment is shown in Fig. 7. The mean phase advance or delay in retinal fragments was smaller, but not statistically different from the mean phase shifts observed in intact eyes presented light pulses at the same phases of the ocular rhythm (intact $= +1.5$ h, 0.9 SD, $n=6$ at CT 2000 and -2.0 h, 0.82 SD, $n=4$ at CT 1200).

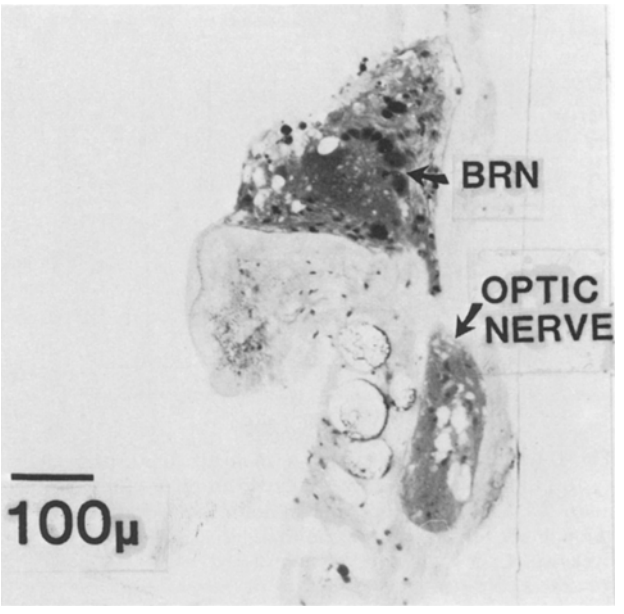

Fig. 4. Thick section of *Bulla* retina following removal of the photoreceptor layer. The remaining tissue fragment consisted of the basal retinal neuron population and associated neuropil

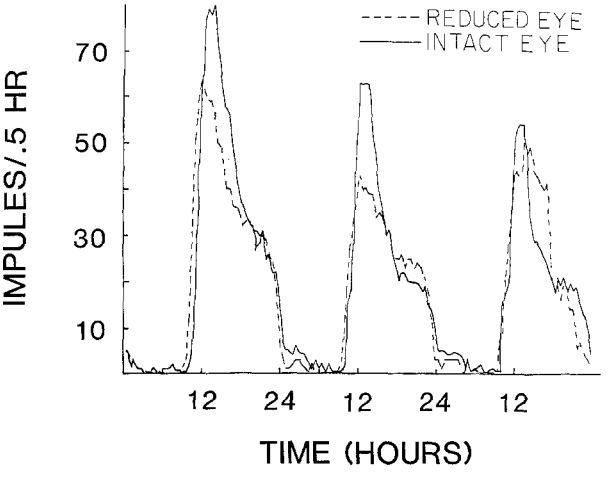

Fig. 5. Ocular circadian rhythms from intact (solid line) and surgically reduced (dashed line) retinas. The photoreceptor layer of the reduced retina was removed prior to recording. Both eyes maintained in continual darkness during recording. Abscissa: clock time in hours (EST). Ordinate: number of spontaneous compound action potentials. Previous dawn was at 09:00

Fractionation of the basal retinal neuron population

As part of this study we sought to determine whether the entire basal retinal neuron population was required for the expression of the circadian rhythm. Eyes in which the photoreceptor layer had been removed were further reduced by cutting deeply into the retinal base. This procedure removed portions of the basal retinal neuron population as well as part of the centrally located neuropil. Approximately 30% of all retinas reduced in this fashion failed to generate optic nerve impulse activity and were discarded. We were successful in producing 32 electrically active retinal fragments. In our smallest fragments we found no evi-

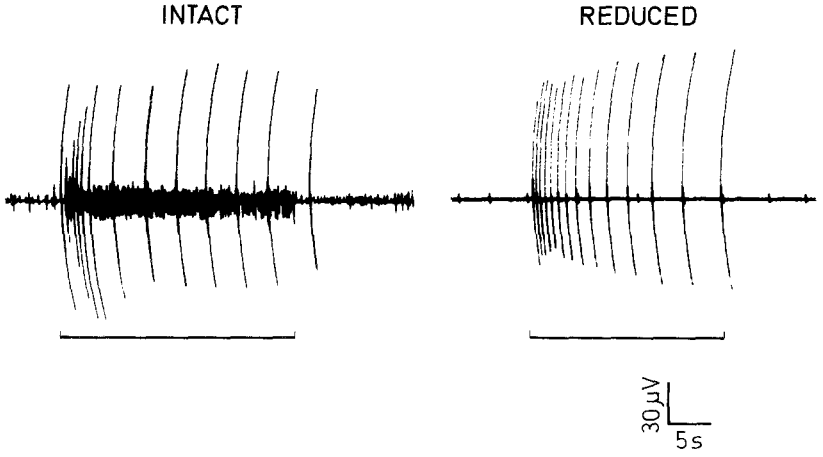

Fig. 6. Light-induced optic nerve responses in intact and surgically reduced eyes. Responses were obtained from eyes maintained in darkness and then exposed to a brief light pulse (15 s, 5000 lux)

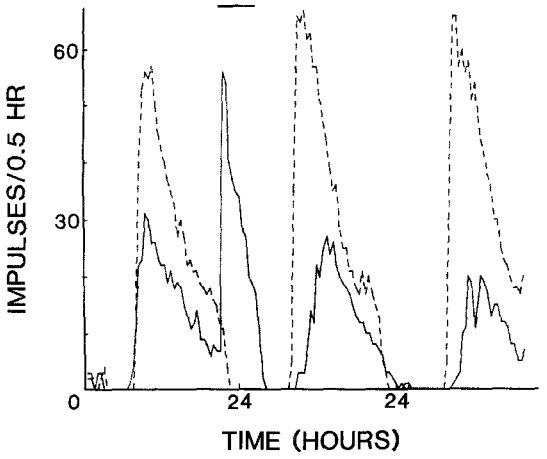

Fig. 7. Phase shift of reduced eye by light. The reduced eye (solid line) was given a 6 hour light pulse at the time indicated by the black horizontal bar (Circadian Time 1200–1800). Onset of light caused a strong sensory response recorded as a sharp peak in impulse frequency. The net phase shift was calculated by comparing the phase of the midrise point of the shifted and control eye (dashed line) on the circadian cycle following the light pulse. Light pulse intensity approximately 5000 lux

dence that neuronal somata remained, while our largest fragments contained from 30 to 35 somata of the approximately 100 cells which typically constitute the basal retinal neuron population.

Ten out of the 12 retinal fragments with more than 15 basal retinal neuron somata remaining continued to exhibit clear circadian periodicities. When fewer than 15 BRN nuclei remained, only two of nine fragments remained rhythmic. Four sample records from retinal fragments containing from 6 to 25 BRN somata are shown in Fig. 8. In general, the circadian rhythms recorded from these severely reduced eyes exhibited more variable waveforms than the rhythms recorded from intact retinas. Also, some of the severely reduced eyes displayed unusually low levels of spontaneous activity. Nevertheless, we observed robust circadian periodicities when as few as 6 BRN somata remained within the ocular fragment (Fig. 8D). Several of the retinal fragments appeared to exhibit hourly oscillations in impulse frequency. These oscillations were most obvious on the second and subsequent cycles of impulse activity when overall activity levels were extremely low (e.g. Fig. 8B). One thick section from serial sections of the retinal fragment containing 6 BRN nuclei is shown in Fig. 9. As can be seen from the photomicrograph all that remained in these tissue fragments were a few basal retinal neuron nuclei and a small portion of the neuropil.

If the retina was reduced until all BRN somata had been removed and only a small amount of neuropil remained, ocular fragments continued to generate compound action potentials. However, there was no clear evidence for a circadian rhythm in impulse frequency. Figure 10 shows three examples of optic nerve impulse frequency records from three fragments lacking BRN nuclei. The upper two panels are typical of ten of the eleven ocular fragments which contained a small amount of neuropil but no BRN somata. Spontaneous impulse activity fluctuated from hour to hour but there was no evidence of a circadian modulation. The record in the lower panel was unique in that it showed some evidence of a low amplitude, low frequency modulation. However, if a rhythm is present, it does not bear the expected phase relationship with the previously applied light cycles (peak activity near projected dawn).

The results of all of our tissue reduction experiments are summarized in Table 1. As can be seen from the table, nearly all ocular fragments with more than 15 BRN somata continued to express a circadian rhythm, while 2 of 9 ocular fragments

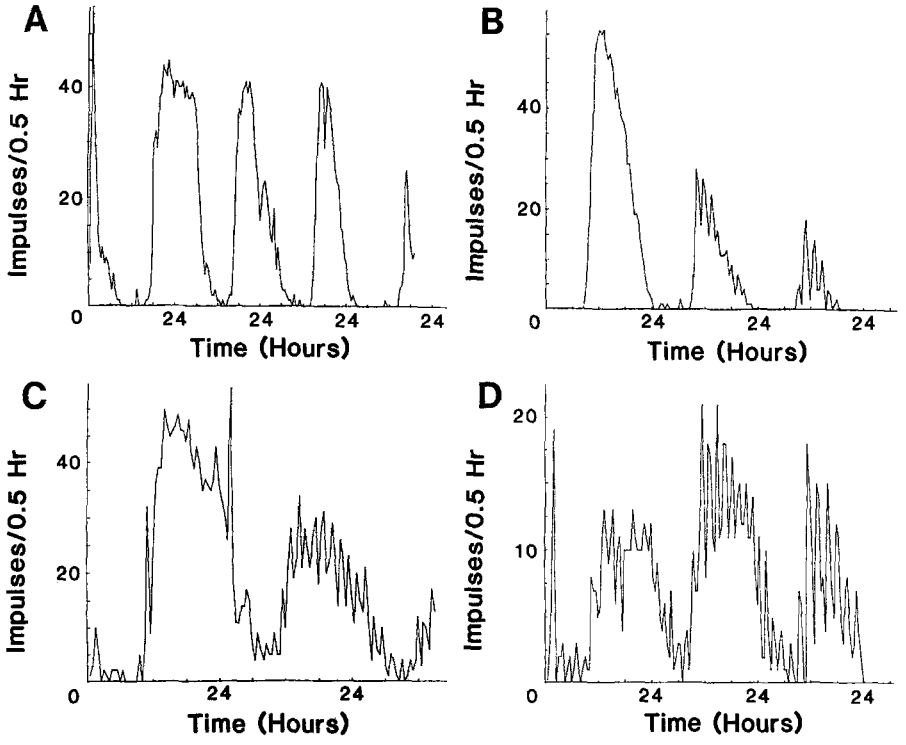

Fig. 8A–D. Four examples of ocular circadian rhythms from retinas with reduced numbers of basal retinal neurons. The estimated number of basal retinal neurons remaining in each fragment is as follows: **A** 25; **B** 20; **C** 15; **D** 6. Prior dawn for **A** was 21:00, dawn was at 09:00 for **B–D**

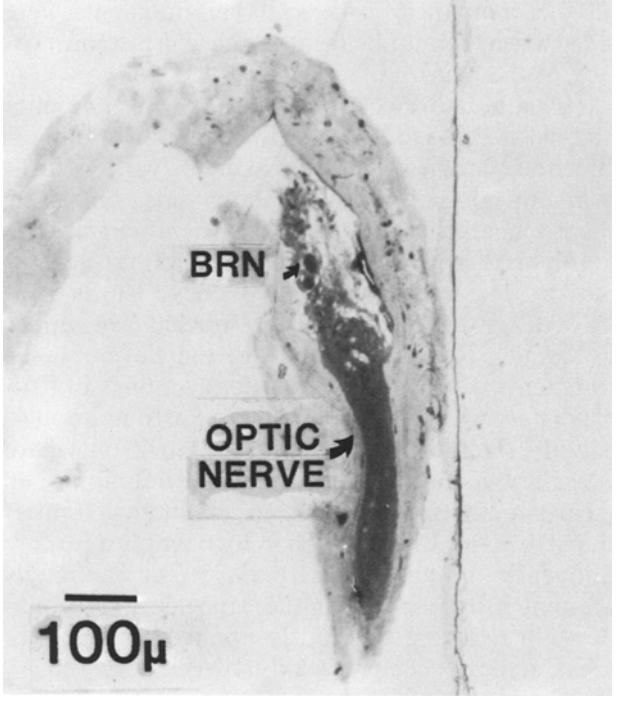

Fig. 9. Thick section of retinal fragment whose activity rhythm is shown in Fig. 8D. This section through the optic nerve and remaining neuropil reveals 2 of the 6 remaining nuclei in this small retinal fragment

with less than 15 BRN somata remained rhythmic. Importantly, the free-running periods of all of the rhythmic ocular fragments were indistinguishable from control eyes.

Discussion

Two conclusions can be drawn from the foregoing results. First, only a few cells at the base of the retina are required for generation and expression of a circadian rhythm in optic nerve impulse frequency. Second, cells in the photoreceptor layer are not required for phase shifts of the ocular pacemaker. Thus a competent circadian pacemaker system resides among cells at the base of the retina. A similar conclusion has been drawn from the results of long-term intracellular recording from retinal cells (Block and Wallace 1982; McMahon et al. 1984).

The results of the present study indicate that as few as 6 basal retinal neurons are sufficient for the expression of a circadian rhythm. However, most retinal fragments containing less than 15 BRN somata were not rhythmic (Table 1). We cannot be certain why some retinal fragments remained rhythmic while others containing a larger proportion of the BRN population were aperiodic.

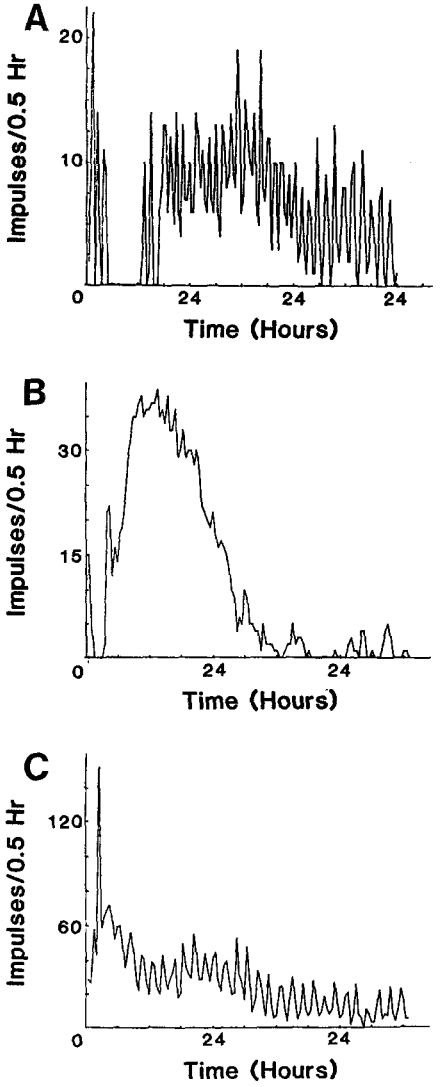

Fig. 10A–C. Spontaneous impulse frequency records from three ocular fragments lacking basal retinal neuron somata. Previous dawn was 09:00

This may indicate the presence of critical circadian pacemaker cells located among the basal retinal neuron population. We think it is more likely, however, that extensive damage to the central neuropil, which was obvious in most of these severely reduced eyes, electrically disconnected many BRN somata from their processes in the optic nerve. We also suspect that damage to the neuropil in severely reduced eyes was responsible for the increased variability in the waveform of the rhythm, and in some eyes, the hourly fluctuations of impulse activity. Alternately, changes in the waveform of reduced eyes may reflect underlying differences in the circadian properties of the BRN population vis-a-vis small groups of basal retinal neurons. However, we believe the salient point is that very few cells

Table 1. Effects of retinal tissue reduction on the ocular circadian rhythm

Number of BRN nuclei remaining in tissue	Percentage of fragments expressing rhythm	*N*	Period/Std Dev
Intact retina	100	10	23.7±0.5 h
Receptor layer removed	100	10	23.9±0.4 h
15 to 30	83	12	23.6±0.5 h
2 to 14	22	9	23.8±0.3 h
0	0	11	–

are required for the expression of a circadian periodicity.

An important finding of the current study is that the free running periods of all reduced and intact retinas were similar (Table 1). It has been proposed for *Aplysia* that the circadian rhythm in impulse frequency emerges from the interactions of a population of non-circadian pacemakers (Jacklet and Geronimo 1971). This conclusion was based upon alleged changes in the free-running period of *Aplysia* eyes which were surgically reduced. This hypothesis has been challenged by Sener (1972) and Strumwasser (1973) based on similar surgical reduction experiments.

We find little evidence in *Bulla* to support the notion that circadian periodicities emerge from circuit interactions among large groups of cells. In *Bulla,* either the circadian pacemaker resides within individual basal retinal neurons, or is due to interactions among a very small group of cells. Since the electrical properties of the basal retinal neuron population appear homogeneous (Block et al. 1984) we consider it most likely that individual basal retinal neurons are circadian oscillators. We must be cautious in stating this hypothesis, however, since we have not yet recorded from isolated basal retinal neurons and we also cannot entirely rule out a role for glial cells in rhythm generation. Glial processes can be seen in close apposition to basal retinal neuron processes in thin retinal sections (Jacklet and Colquhoun 1983). One argument against a role for glia is the observation that the ocular rhythm is disrupted by removal of BRN somata, even though glial cell nuclei are still obvious in some reduced retinas. As we have emphasized, an unambiguous demonstration of the sufficiency of basal retinal neurons as circadian pacemakers will require the physical isolation of individual neurons.

A second conclusion we draw from the results of the present study is that cells at the base of

the retina are capable of light transduction leading to phase shifts in the ocular pacemaker (Fig. 7). Previous experiments with localized illumination demonstrate that neurons at the base of the retina are light sensitive (Block and McMahon 1983; Block et al. 1984). There is no morphological evidence for photoreceptor specializations at the base of the retina (Jacklet and Colquhoun 1983), although, many molluscan neurons exhibit light sensitivity (Arvanitaki and Chalazonitis 1960).

While we observed both light-induced phase advances and delays in retinal fragments in which the photoreceptor layer was removed, the mean phase shifts were somewhat smaller in the fragments when compared to intact retinas. This may indicate some role for the photoreceptor layer in entrainment of the ocular pacemaker. Cells in the photoreceptor layer have been shown to influence basal retinal neurons (Block et al. 1984). Unfortunately, the small sample sizes and rather large standard deviations for phase shifts in this experiment preclude any confident assessment about whether the differences in the magnitude of the light-induced phase shift of intact and reduced eyes are real. This is an interesting issue which deserves further study.

Tissue reduction experiments also provide evidence that the site for the initiation of spontaneous impulses and the circadian pacemaker locus in the *Bulla* retina are anatomically discrete. Eyes in which all BRN somata were removed continued to generate compound action potentials as long as a small amount of neuropil remained attached to the optic nerve.This indicates that spontaneous spike initiation and the electrical coupling of basal retinal neurons to produce impulses occurs outside the soma, presumably in the neuropil. This view is consistent with the ultrastructural findings of Jacklet and Colquhoun (1983) who report the absence of gap junctions between the closely apposed somata of neurons in the base of the eye, but a large number of these junctions in the neuropil. In *Aplysia,* the functional separation of the spontaneous impulse initiating region and the circadian pacemaker system is supported by experiments from Strumwasser's laboratory where it was found that ionizing radiation abolishes the circadian rhythm without affecting the spontaneous production of optic nerve impulses (Woolum and Strumwasser 1980), and by experiments using protein synthesis inhibitors where the circadian rhythm was blocked without affecting spontaneous impulse activity (Rothman and Strumwasser 1977; Jacklet 1980). In addition, Jacklet and Geronimo (1971) found that some *Aplysia* eyes which were surgically reduced continued to generate impulses without evidence of a circadian periodicity.

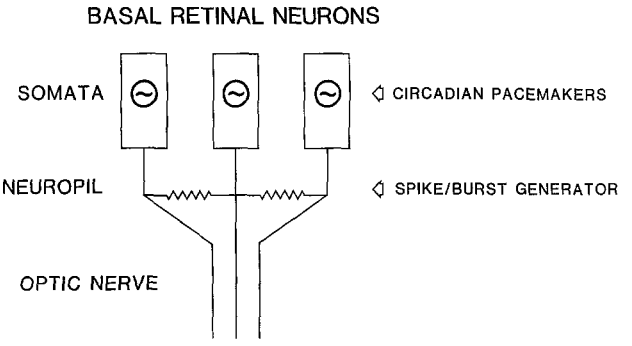

Fig. 11. Proposed model for the organization of the circadian pacemaker system in *Bulla*. See text for details

On the basis of electrophysiological studies (Block et al. 1984; McMahon et al. 1984), morphological information (Jacklet and Colquhoun 1983; Block and Wallace 1982), and tissue reduction experiments, we have formulated a 'working hypothesis' about the organization of the circadian pacemaker system in the *Bulla* retina (Fig. 11). We think it is likely that each basal retinal neuron is a competent circadian oscillator. Thus, there are approximately 100 oscillators in each retina. Furthermore, we believe that the circadian pacemaker function resides in the soma of each basal retinal neuron and modulates, via membrane potential, a spontaneous impulse generating mechanism located in the neurite. Considering the entrainment of the ocular circadian pacemaker, we envision three possible pathways: from phototransduction occurring within each basal retinal neuron, through efferent fibers from the contralateral eye (Roberts and Block 1983) and perhaps through synaptic contacts with H-type photoreceptors (Block et al. 1984).

The proposed model generates several important, testable hypotheses. If a somatic circadian pacemaker is responsible for modulating an extrasomatic action potential generating mechanism, then BRN somata which are severed from their connections with the neuropil should express a circadian rhythm in membrane potential, although not generate spontaneous action potentials. Preliminary experiments reveal that basal retinal neurons are uncharacteristically silent after the optic nerve and part of the neuropil region have been removed. We have not yet recorded long-term from these cells, however, to ascertain whether or not a membrane potential rhythm is present. Another prediction of our model is that once the neuropil region has been destroyed, each BRN soma

should exhibit an independent circadian rhythm in membrane potential. Thus, it should be possible to record multiple circadian rhythms within a single, surgically reduced retina. This is a difficult, but feasible experiment.

Our model also raises fundamental questions about the role of BRN membrane potential in the generation and control of circadian rhythmicity within the eye. If we are correct in our assertion that basal retinal neurons are autonomous circadian pacemaker cells, then membrane potential may serve two functions, first as an input pathway to transfer entrainment signals to the cell, and also as an output pathway – the means by which the BRN soma modulates impulse frequency (see Strumwasser et al. 1979). The importance of membrane potential changes in the entrainment of the *Aplysia* eye has been carefully documented (Eskin 1977, 1982). The critical question remains whether membrane potential plays a passive role in transferring information to and from an intracellular circadian oscillator or whether it is actually part of the causal loop generating the circadian oscillation (i.e., a state variable in the oscillator). The ability to perform detailed intracellular electrophysiological experiments on neurons in the *Bulla* retina provides a special opportunity to address these important issues.

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