ty, presumably because of the occurrence of presynaptic action potentials. Records from one such cell are shown in Fig. 2. The upper trace illustrates the response of the cell to glycine; the second trace, obtained at a higher gain, shows the spontaneous activity. The bulk of the spontaneous synaptic currents are inward (downward deflections), corresponding to excitation, but several large inhibitory events (upward deflections) can also be seen. The numbered segments are expanded on the four subsequent traces to show the time course of the inhibitory currents. Strychnine abolished the response to glycine in these cells (upper trace in Fig. 2B) (*5*) and the spontaneous inhibitory currents as well (lower trace). A total of 44 IPSC's from four different cells had amplitudes that were distributed unimodally, with a mean corresponding to a peak conductance of 107 ± 62 nS, and decayed exponentially with a mean time constant of 32 ± 11 msec, the same as the mean relaxation time obtained for the glycine-activated channels. The average reversal potential for the IPSC's was −67 mV and, in individual cells, was indistinguishable from the reversal potential for the responses to glycine.

In summary, both the glycine responses and the IPSC's were blocked by strychnine, both had the same reversal potential, and the time constant of decay of the IPSC's was identical to the mean relaxation time constant of the glycine-activated channels. These observations support glycine as the inhibitory transmitter in these cells. The channel conductance reported here is two to five times greater than that reported for chloride channels in other cells (*1*), but is consistent with the large conductance underlying the IPSC's. Thus the spontaneous inhibitory events each appear to be associated with the synchronous activation of about 1500 channels, which is similar to the number of excitatory channels activated by a quantum of acetylcholine at the neuromuscular junction of the frog (*6, 7*). This, in turn, suggests that the spontaneous IPSC's are due to the release of single quanta of inhibitory transmitter. In support of this idea, we have preliminary evidence that spontaneous IPSC's produced in tetrodotoxin-blocked preparations by potassium depolarization have equivalent amplitudes.

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Mutual Coupling Between the Ocular Circadian Pacemakers of *Bulla gouldiana*

Abstract. *The ocular circadian pacemakers of* Bulla gouldiana *were found to be mutually coupled, and their interaction could be observed in an isolated nervous system maintained in vitro. Experimentally induced phase separations between the two ocular pacemakers were reduced when the eyes were allowed to interact for 48 hours. The reduction in phase separation did not occur however when the cerebral commissure was severed, indicating that this neural tract is a critical pathway coupling these two circadian clocks.*

Most circadian behaviors analyzed to date are under the control of multiple pacemakers (*1*). For example, Page (*2*) has described the effects on locomotor behavior of shifting the phase of one member of a bilaterally distributed pacemaker pair in the cockroach circadian system. These elegant experiments, and the work of others (*3*), indicate that, in the cockroach, locomotor rhythmicity is controlled by two mutually coupled circadian pacemakers located in the optic lobes. Similarly, in the rodent, the dissociation of the locomotor rhythm into two independent free-running components ("splitting") suggests that at least two pacemakers control locomotor activity in these animals (*4*). Because of the apparent multi-oscillator nature of circadian systems, a complete understanding

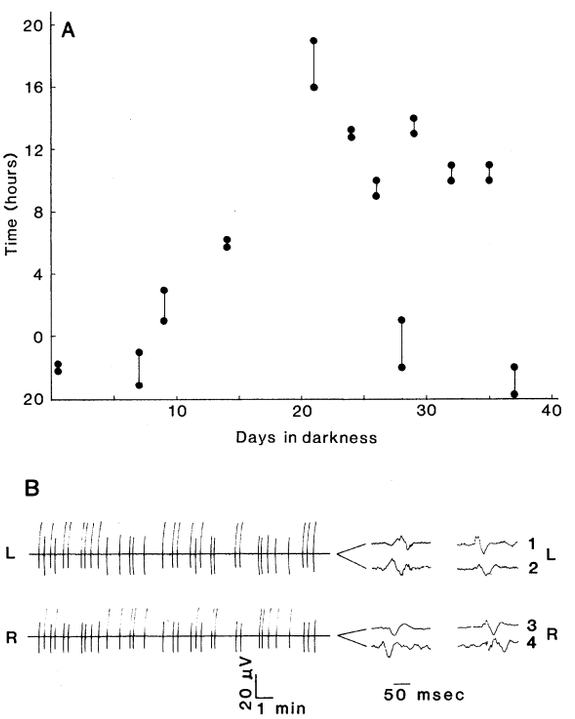

Fig. 1. (A) Phase separations in vivo between the ocular pacemakers of *Bulla*. After a specified time in darkness, both eyes of *Bulla* were removed and the ocular rhythms recorded. Each vertical line in the figure connects the phase reference points of the two ocular rhythms from a single animal. The ordinate shows the time of day (old dawn, 0 hours). As the time in darkness increases, the two ocular rhythms drift relative to clock time (that is, the eyes are free-running), but maintain a relatively constant phase relation to one another, never deviating by more than 3 hours [mean phase separation, 1.45 ± 1.03 (S.D.) hours.] (B) (Left) Recording from both optic nerves of an intact nervous system maintained in vitro. Each optic nerve potential is followed one for one by an efferent signal in the contralateral optic nerve. (Right) Oscilloscope recordings from an isolated *Bulla* nervous system, made with four electrodes, two on each optic nerve: (traces 1 and 2), left optic nerve; (traces 3 and 4), right optic nerve. Electrodes 2 and 3 are closest to the cerebral ganglia. The temporal ordering of the signals (4-3-2-1 or 1-2-3-4) demonstrate that the impulses pass down one optic nerve and up the contralateral optic nerve.

of circadian organization requires detailed analysis of pacemaker interactions (*5*).

Because an activity rhythm represents an output of the entire circadian system, the exact state of each contributing pacemaker is difficult to assess. We now show that the marine mollusc *Bulla gouldiana* has two mutually coupled circadian pacemakers whose interaction can be observed and manipulated in an isolated nervous system maintained in vitro.

As in *Aplysia*, a related gastropod mollusc, both *Bulla* eyes contain pacemakers that drive a circadian rhythm in the frequency of spontaneously occurring optic nerve impulses (*6*). The pacemakers are located among a small group of cells at the base of each retina (*7*). *Bulla* also exhibit a circadian locomotor rhythm. Eye removal leads to aperiodic locomotor behavior, suggesting that the ocular pacemakers play a clock-like role in timing the activity rhythm (*8*).

Two observations suggested to us that the *Bulla* ocular circadian pacemakers are coupled. First, animals maintained in continual darkness for 1 month or more during the course of locomotor experiments had ocular rhythms that were almost in phase with one another. Further analysis of ocular phase separations after periods of darkness confirmed this observation (Fig. 1A). This result is surprising since, in *Aplysia*, the phases of the ocular rhythms are separated by as much as 9 hours after only 3 weeks in continual darkness (*9*). Second, in recordings made from both optic nerves of intact *Bulla* nervous systems in vitro, afferent impulses generated by one eye were followed one for one by efferent impulses in the contralateral optic nerve (Fig. 1B). Thus the output of each ocular pacemaker is available to the other retina.

Three criteria should be met in order to demonstrate coupling between two circadian pacemakers. First, the pacemakers should maintain a stable phase relation in the absence of external synchronizing cues. Second, experimentally induced desynchrony should lead to the reestablishment of a stable phase relation through pacemaker interactions. Finally, if the coupling between the two pacemakers is mutual, as distinguished from hierarchical, synchrony should be regained through transient phase shifts of both pacemakers' rhythms (*10*).

According to the above criteria, experimentally induced interocular desynchrony should be minimized or eliminated through pacemaker interactions if the ocular pacemakers are coupled. Experimentally shifting the phase of one of the pacemakers would then allow the effects of this desynchrony on the behavior of the two ocular rhythms to be observed. Experiments were performed with isolated central nervous systems, which were placed in specially designed treatment chambers (Fig. 2A). Phase shifts were induced by pulsed application to one eye of an artificial seawater solution in which calcium was replaced with manganese (*11*).

We first determined the magnitude of the Mn-induced phase shift by isolating and recording the ocular rhythms of some of the eye pairs ($N = 7$) immediately after the Mn treatment (*12*). We then tested for the effects of possible coupling by leaving the rest of the eye pairs ($N = 10$) attached to the nervous system and thus free to interact for 48 hours after the Mn pulse. We found that the Mn initially produced about a 5-hour phase difference between the treated and untreated eyes [mean ± standard deviation (S.D.), 4.71 ± 0.76]. This difference was reduced to 2 hours (1.95 ± 0.69) in the eye pairs that were allowed to interact (*INT* in Fig. 2B). The observed reduction in phase separation occurs through transient phase advances in the initially shifted ocular rhythms and phase delays in the initially unshifted ocular rhythms (Fig. 2C) (*13*). The final mean phases of eye pairs after 48 hours of interaction (*INT*) was significantly different from the mean phases of eyes prevented from interacting for the 48-hour period (*CCX* in Fig. 2; see below).

These results suggest that pacemaker-pacemaker interactions lead to the tran-

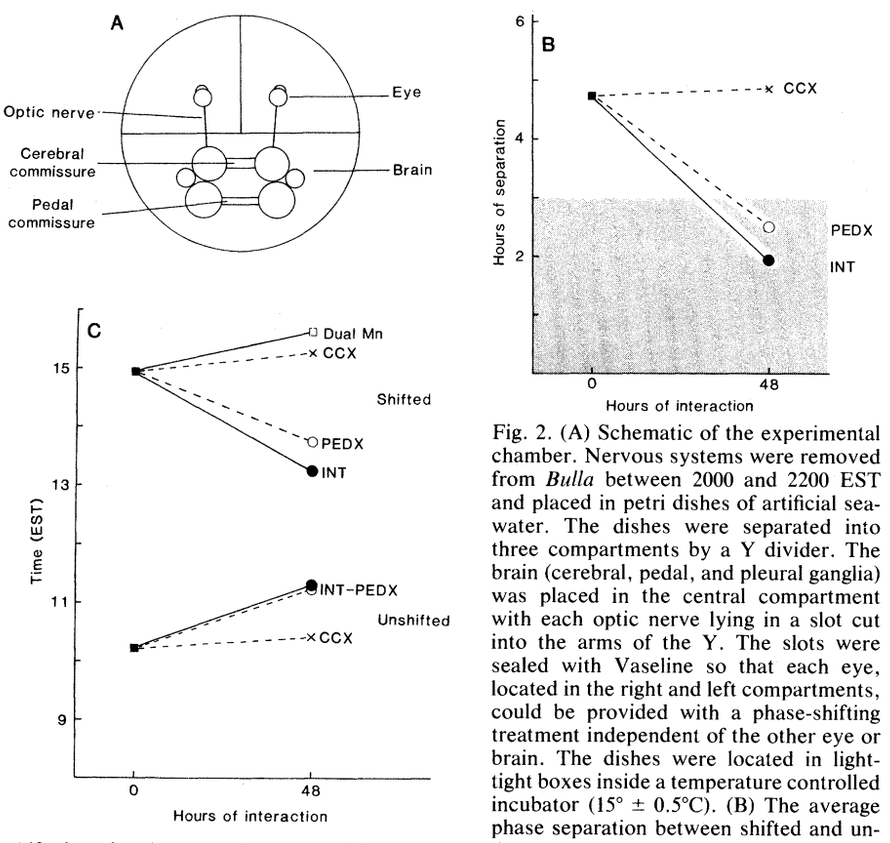

Fig. 2. (A) Schematic of the experimental chamber. Nervous systems were removed from *Bulla* between 2000 and 2200 EST and placed in petri dishes of artificial seawater. The dishes were separated into three compartments by a Y divider. The brain (cerebral, pedal, and pleural ganglia) was placed in the central compartment with each optic nerve lying in a slot cut into the arms of the Y. The slots were sealed with Vaseline so that each eye, located in the right and left compartments, could be provided with a phase-shifting treatment independent of the other eye or brain. The dishes were located in light-tight boxes inside a temperature controlled incubator (15° ± 0.5°C). (B) The average phase separation between shifted and unshifted ocular rhythm pairs recorded immediately after the manganese pulse is displayed as a filled square. The phase separation between shifted and unshifted eye pairs that were allowed to interact for 48 hours is displayed as a filled circle (*INT*). Severing the cerebral commissure (*CCX*) blocks the reduction in phase separation, whereas section of the pedal commissure (*PEDX*) appears to have little effect. The gray band displays the range of phase separations between the ocular rhythms recorded in our studies in vivo. (C) The average half-maximum phase reference points for all eyes are plotted in relation to clock time. Reference points for eyes isolated immediately after the manganese pulse are plotted as 0 hours of interaction (solid square). The unshifted eyes show half-maximum points near projected dawn (9:00 EST), whereas the shifted eyes have been delayed. After 48 hours of interaction (*INT*; solid circles and solid lines), the phase separation has been reduced through the movement of both the shifted and the unshifted eye rhythms toward an intermediate phase. These transients are not observed if the interaction is blocked by cerebral commissure section (*CCX*; small crosses and dashed lines), while section of the pedal commissure (*PEDX*; open circles and dashed lines) appears to have little effect on the reduction in phase separation. Open squares and solid lines indicate phase reference points of eye pairs that were both subjected to phase delay by manganese treatment (*Dual Mn*); see text.

sient phase shifts and the eventual adoption of an intermediate phase by the two ocular rhythms. We were concerned, however, that the phase advances in the Mn-treated eyes during the 48 hours of interaction might be an artifact of the chemical treatment and unrelated to the desynchrony of the two ocular rhythms. If this were the case, initially shifting both pacemakers with Mn, thereby maintaining ocular synchrony, would still lead to phase advances in the treated eyes during the 48-hour interaction period. The results indicate, however, that without desynchrony, phase advances do not occur. When both pacemakers were treated with Mn (*Dual Mn* in Fig. 2C), the initial phase delay was maintained (*14*). Thus, the phase separation between the two pacemakers, and not the Mn treatment as such, generates the phase advances that lead to a reduction in phase separation between the two ocular rhythms.

The two possible neural pathways by which the ocular pacemakers can interact are the cerebral commissure and the pedal commissure. Cutting the pedal commissure (*PEDX* in Fig. 2; $N = 4$) had no effect on the reduction in phase separation, whereas cutting the cerebral commissure (*CCX*; $N = 6$) blocked the reduction in phase separation normally observed after pacemaker interaction (Fig. 2, B and C). This indicates that the cerebral commissure is a critical pathway for pacemaker coupling (*15*).

The ocular pacemakers of *Bulla gouldiana* meet the criteria needed to show mutual coupling. First, our experiments in vivo reveal that the pacemakers' rhythms remain closely synchronized in the absence of temporal cues. Second, if the eyes are allowed to interact in vitro, experimentally induced phase differences are reduced to the range of values observed in the studies in vivo. Finally, our experiments show that both ocular rhythms undergo transient phase shifts in the course of their interaction.

Thus, we have shown mutually coupled neural circadian pacemakers in vitro (*16*). The identification of a critical coupling pathway and the ability to manipulate this system in vitro should provide opportunities for studying the physiology of interacting pacemakers and the underlying entrainment processes.

The fact that the *Bulla* ocular pacemakers are coupled whereas their *Aplysia* counterparts interact only weakly is surprising in view of the similarities between the animals' circadian systems. An explanation may be found in the different behavioral niches occupied by these animals. *Aplysia* are diurnal and are continually exposed to the environmental light cycle. *Bulla*, in contrast, are nocturnal and spend the day buried in the sand. It has been suggested that in general, diurnal animals can rely on daily environmental time cues to maintain pacemaker synchrony, whereas nocturnal animals, which may not detect the light cycle for a number of days, require tight coupling between their circadian pacemakers in order to avoid desynchrony (*17*). Thus, strong pacemaker coupling may have evolved in *Bulla* in order to maintain synchrony in its circadian system.

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10. Depending on the underlying phase response curves for the coupling interaction, there may be some phase separations between two pacemakers that result in only one of the two rhythms shifting toward the stable phase.
11. It has been shown by A. Eskin and G. Corrent [*J. Comp. Physiol. A* **177**, 1 (1977)] that pulse application of Mn-substituted seawater shifts the phase of the *Aplysia* eye clock. In our experiments, Mn pulses were given by replacing the artificial seawater (ASW) bathing one of the eyes with four changes of Mn seawater (MnASW; NaCl, 395 m*M*; KCl, 10 m*M*; $MgCl_2$, 50 m*M*; $NaSO_4$, 28 m*M*; $MnCl_2$, 15 m*M*; Hepes, 30 m*M*; penicillin, 100,000 U/liter; and streptomycin, 100,000 μg/liter). The MnASW was removed by replacing the solution with six changes of ASW. Eyes were treated with MnASW from 1500 to 2100 EST.
12. Because impulse activity in one eye inhibits impulse production in the contralateral eye, the wave form of the rhythms from interacting eyes cannot be used as an unambiguous reference for the phase of each pacemaker. Therefore it is necessary to record the ocular rhythms with the eyes isolated from the nervous system to accurately assess the phase of each pacemaker's rhythm. For recording, eyes were placed in petri dishes filled with artificial seawater (Instant Ocean; plus Hepes, 30 m*M*; penicillin, 100,000 U/liter; and streptomycin, 100,000 μg/liter), with the optic nerves in glass suction electrodes. Optic nerve activity was led off with silver wire, amplified, and displayed on a polygraph (Grass). Optic nerve impulses were counted into half-hour intervals, with the half-hour closest to one-half maximum frequency chosen as the phase reference point for that rhythm.
13. Mean ± standard error (S.E.M.) of phase reference points (all times, EST) at zero hours of interaction, for shifted eyes, 14.93 ± 0.39 hours, and for unshifted eyes, 10.21 ± 0.31 hours. After 48 hours of interaction, the value for shifted eyes was 13.25 ± 0.30 hours, and for unshifted eyes, 11.30 ± 0.30 hours.
14. Phase reference points for treatment of both eyes with Mn, 15.62 ± 0.80 (S.E.M.) hours ($N = 4$).
15. Phase reference points for pedal commissure section: for shifted eyes, 13.75 ± 0.66 (S.E.M.) hours, and for unshifted eyes, 11.25 ± 0.76 (S.E.M.) hours; mean ± S.D. of phase difference, 2.5 ± 0.41 hours. Phase reference points for cerebral commissure section: for shifted eyes, 15.23 ± 0.43 (S.E.M.) hours, and for unshifted eyes, 10.42 ± 0.43 (S.E.M.) hours; phase difference, 4.83 ± 0.93 (S.D.) hours. The final mean phases of ocular rhythms from nervous systems with cut cerebral commissures was significantly different from the final mean phases of ocular rhythms from intact nervous systems after 48 hours of interaction (shifted, $P < .005$; unshifted $P < .025$).
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