Light and serotonin interact in affecting the circadian system of *Aplysia*

Christopher S. Colwell

Department of Biology, University of Virginia, Charlottesville, VA, 22901, USA

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**Summary.** The eye of the marine mollusk *Aplysia californica* contains a photo-entrainable circadian pacemaker that drives an overt rhythm of spontaneous compound action potentials. The current study evaluated the influence of serotonin on light-induced phase shifts of this ocular rhythm. The application of serotonin in combination with light was found to have profound and interactive effects on the magnitude of the resulting phase shifts. Further, the phase shifts that resulted from the interaction between light and serotonin appeared to be phase dependent, i.e., the application of serotonin inhibited the phase shifting effects of light during one part of the circadian cycle but enhanced them during another. Finally, the results show that the interaction between light and serotonin is dependent upon the sequence in which these two treatments are paired. These data, coupled with previous findings, suggest that serotonin may act to modulate light's phase shifting effects on the ocular pacemaker in *Aplysia*.

**Introduction**

A defining feature of circadian oscillations is that they can be synchronized (entrained) to periodic signals from the environment. The daily light-dark cycle is an environmental cue which entrains all known circadian oscillators. However, the effects of light signals on circadian pacemakers may be influenced by efferent regulation as well as other factors such as the hormonal state of the organism. In order to fully understand entrainment, it is important to characterize and understand the impact of such variables on light-induced phase shifts of circadian oscillations.

The eye of the marine mollusk *Aplysia californica* contains a circadian pacemaker that drives an overt rhythm of spontaneous compound action potentials. This rhythm can be entrained in vitro by light cycles (Eskin 1971). Several pieces of evidence suggest that serotonin (5-HT) may also be involved in regulating entrainment of the circadian pacemaker in the eye. First, the eye contains 5-HT and is capable of synthesizing it from tryptophan (Corrent and Eskin 1982). Furthermore, anatomical studies have demonstrated that 5-HT-like immunoreactivity can be localized to efferent fibers which project to the eye from the cerebral ganglion (Goldstein et al. 1984; Olsen and Jacklet 1985; Takahashi et al. 1989). Finally, exogenously administered 5-HT (10⁻⁶ M) and analogs can phase shift (both advance and delay) the rhythm (Corrent et al. 1982).

However, the relationship between the effects of light and those of 5-HT is not simple. Although this transmitter can produce both advances and delays in the rhythm expressed by isolated eyes in vitro, it does not mimic the effects of light. In fact, the phase response curves of light and 5-HT seem to be displaced relative to one another by 12 h (i.e., at phases at which light produces a phase advance, 5-HT tends to produce a phase delay and vice versa; Corrent et al. 1982). Moreover, 5-HT is not acting as a neurotransmitter in the light entrainment pathway of the ocular pacemaker because 5-HT causes phase shifts during phases at which light does not, and synaptic transmission does not appear to be required for light-induced phase shifts (Eskin 1977). Thus 5-HT can apparently act on the circadian system in the isolated eye through a phase shifting pathway distinct from that of light's.

Results from previous studies are also consistent with the hypothesis that 5-HT can act to modulate the effects of light on the circadian system. The presence of efferent fibers innervating the eye was first reported by Eskin (1971) who later observed that electrical stimulation of the nerve which contains these fibers acted to increase the photosensitivity of the eye (Eskin and Maresh 1982). In addition, in several experimental paradigms, light's effect on the phase of the ocular rhythm was influenced by efferent activity (Block et al. 1974; Block and Page 1978;
Pritchard and Lickey (1981). In at least one of these cases, application of 5-HT was shown to mimic the effect of efferent activity (Nadakavukaren et al. 1986). Importantly, none of these experiments directly investigated the effects of 5-HT on light-induced phase shifts.

The purpose of the present study was to examine whether 5-HT could act to regulate the effects of light on this circadian system. Specifically, we sought to determine whether the bath application of 5-HT could influence light-induced phase shifts in the circadian rhythm recorded from the isolated eye of *Aplysia*.

**Materials and methods**

*Aplysia californica* were obtained from Alacrity Marine Supply, Redondo Beach, California, and maintained in artificial seawater (ASW) at 15 °C. Animals were entrained to a light-dark cycle (LD 12:12) for at least one week prior to experimental setup. Two hours before the onset of darkness animals were immobilized with an injection of isotonic MgCl₂ and then dissected.

For extracellular recordings, both eyes with their optic nerves were removed from each animal and placed in separate dishes of filtered (0.22 µm, Millipore) ASW. The composition of ASW was (in mM) 395 NaCl, 10 KCl, 10 CaCl₂, 30 MgCl₂, 28 Na₂SO₄, 30 Hepes buffer, 100,000 unit penicillin and 100,000 µg streptomycin. The optic nerve was sucked into a polyethylene tube embedded in Sylgard (Dow Corning). A silver wire was inserted into the tube containing the nerve. The recording dish was placed in a light-tight recording chamber and maintained at 15 °C for the duration of the experiment. A Grass polygraph was used to amplify and record the compound action potentials (CAPs). When the number of CAPs are counted into 30 min bins, a circadian rhythm of CAP frequency can be recorded from each eye. In these experiments, one eye from each animal serves as the control for the contralateral (experimental) eye. The phase relationship between the daily peaks of activity for each pair of eyes was determined by comparing the time of occurrence of the half maximum spike frequency on the rising phase of each daily cycle of activity. The time of half maximum of the rhythm was chosen as an assay of the phase, because the variance between rhythms from a pair of eyes is low. For instance, in control experiments in which both eyes from one animal were untreated, the mean phase difference between the time of half maximum of the two rhythms was found to be 0.0 h (standard deviation 0.25, n = 9).

The first cycle of CAP activity from experimental and control eyes was recorded prior to treatment to assess any phase difference between them and to ensure that the rhythms were properly entrained. The experimental treatments were applied prior to the 2nd peak of activity, either in the late subjective day as the CAP activity is decreasing, or in the late subjective night just before and during the rising phase of CAP activity. Because the period of the ocular rhythm is close to 24.0 h, eastern standard time was used as an approximation of circadian time to determine the phase of experimental pulse treatments.

The effects of experimental treatments were calculated by measuring the phase difference on the 3rd cycle (the cycle after treatment), less the phase difference from the first (pretreatment) cycle. Since eyes from the same animal have very similar rhythms, the phase difference of the first cycle was typically small (<0.25 h). The phase shift was complete by the 3rd cycle as the phase shifts measured during the 3rd cycle were very similar to those that could be measured during the 4th. For example, the mean phase shifts produced by 5-HT treatments (10⁻³ M) from CT 6–12 for those experiments which ran at least 5 cycles were +1.58 h in the 3rd cycle and +1.60 h in the 4th cycle (n = 6); the same treatment from CT 18–24 caused phase shifts of −1.28 h in the 3rd cycle and −1.57 h in the 4th cycle (n = 7). Similarly, phase shifts produced by light treatment from CT 6–12 were −1.5 h in the 3rd cycle and −1.3 h in the 4th cycle (n = 6); light treatment from CT 18–24 caused phase shifts of +2.60 h in the 3rd cycle and +2.50 in the 4th cycle (n = 6). Rhythms were recorded for at least 5 days before termination of an experiment to ensure that the phase shifts were stable. Light pulses were delivered by a microscope illuminator positioned above the eyes to deliver an intensity of 60 µW/cm². The temperature in the solutions surrounding the eyes increased by <1 °C during the light pulses.

Solutions of 5-HT were made by adding the drug directly to ASW 2 h before use in experiments. Solution changes were made without illumination of the eyes through use of an infrared viewer. The experimental eyes were rinsed 3 times (50 ml each) with the experimental solutions before remaining in these solutions. At the end of the treatments, the experimental solutions were withdrawn and the eyes were rinsed 5 times (50 ml each) with ASW.

The effects of the treatments were evaluated using either 1-way or 2-way ANOVA, where appropriate, followed either by Scheffe's or Tukey's multiple comparison procedures where appropriate. Values are shown as means ±95% confidence interval and were considered significantly different if P < 0.05.

**Results**

In the first experiment, the effects of 5-HT on the magnitude of light-induced phase delays was examined. A 6 h treatment of light, starting at CT 6, caused a phase delay of 1.4 h (±0.3, n = 6), while a 6 h application of 5-HT at the same phase produced a phase advance of 1.7 h (±0.6, n = 6). When the 5-HT and light treatments were given simultaneously, no phase shifts were observed (0.1 h, ±0.7, n = 6; Fig. 1). Therefore 5-HT appeared to inhibit the effects of light on the ocular rhythm. At CT 6–12, light and 5-HT each produced phase shifts that were approximately equal in magnitude but opposite in sign, and analysis of these data revealed no significant interaction between these two treatments (F = 0.22). At CT 9–15, light treatment produced a phase delay of 2.5 h (±0.4, n = 7), while 5-HT produced a phase advance of 0.9 (±0.7, n = 6). Together, the application of light and 5-HT again resulted in no phase shift of the ocular rhythm (0.1, ±0.7, n = 6). In this case, there was a significant interaction between 5-HT and light (F = 9.94; P < 0.005).

A dose response curve was then constructed for the effects of 5-HT on light-induced phase delays. The experimental eyes received light and 5-HT at CT 6–12 while the control eyes were left in the dark. The concentration of 5-HT was varied from 10⁻⁸ to 10⁻⁴ in log unit steps. The results (Fig. 2), demonstrate that the effects of 5-HT were dose dependent. The ED₅₀ of 5-HT's effect on light-induced phase shifts was approximately 10⁻⁷ M and the response saturated at approximately 10⁻⁶ M.

The effect of 5-HT on light-induced phase advances was also investigated. At CT 18–24, light treatment caused a phase advance of 2.6 h (±0.5, n = 6) and 5-HT caused a phase delay of 1.5 h (±0.4, n = 6). When the treatments were given simultaneously, a phase advance of 4.0 (±0.4, n = 5) was observed (Fig. 3). This phase shift was significantly greater (P < 0.01) than that produced by light alone. Furthermore, there was again a significant interaction between light and 5-HT (F = 59.32; P < 0.0001).
Fig. 1. Plot of CAP activity as a function of time showing that no phase shift resulted when 6 h pulses of light (60 μW) and 5-HT (10^{-5}) were applied simultaneously from CT 6–12. Solid line with open squares is the experimental eye; dashed line with filled diamonds is the control eye from the same animal. Out of range values occurred during treatment.

Fig. 2. Concentration-response curve constructed for the effect of 5-HT on light-induced phase delays. Experimental eyes received light and 5-HT at CT 6–12 while control eyes were left in the dark. Concentration of 5-HT varied from 10^{-8}–10^{-4} in log unit steps. Phase shift induced by light alone is indicated as having a 5-HT concentration of zero. Phase shift values are means given in hours with error bars showing 95% confidence intervals.

Fig. 3. Plot of CAP activity as a function of time showing the phase shift which resulted when 6 h pulses of light (60 μW) and 5-HT (10^{-5}) were applied simultaneously from CT 18–24. Solid line with open squares is the experimental eye; dashed line with filled diamonds is the control eye from same animal. Out of range values occurred during treatment.

Fig. 4. In these experiments, phase of 6 h light pulse remained constant while phasing of 6 h serotonin pulse was varied. Light pulse was given at CT 18–24 while 5-HT pulse was given at either CT 12–18, 15–21, 18–24, or 21–03. Phase shifts which resulted from light alone treatment at CT 18–24 and 5-HT alone treatment from CT 12–18 are shown for comparison. Phase shift values are means given in hours with 95% confidence intervals. Number of paired eyes in parentheses.

The final set of experiments was designed to investigate how the sequence in which the 5-HT treatment was paired with light affected the magnitude of the resulting phase shift. In these experiments, a 6 h 5-HT treatment was given at either CT 12–18, 15–21, or 21–03 in combination with a light treatment from CT 18–24. The results are shown in Fig. 4: the application of 5-HT from CT 12–18 followed by light from CT 18–24 resulted in phase shifts which were significantly larger (P<0.05) than those produced by light alone or those produced by the simultaneous application of light and 5-HT. The application of 5-HT from CT 15–21 in combination with light also caused phase shifts which were also significantly larger (P<0.05) than those produced by light alone but these were not significantly different from those produced by the simultaneous application of these two treatments. Finally, the application of 5-HT from CT 21–03 did not significantly change the magnitude of light-induced phase shifts. These results show that the interaction between light and 5-HT is dependent upon the order in which these two treatments are paired.

The effect of pretreatment with 5-HT on light-induced phase delays was also investigated. A 6 h treatment of 5-HT from CT 0–6 in combination with a light
treatment from CT 6–12 resulted in a phase delay of 1.5 h (± 0.9, n = 5). This phase shift was not significantly different (P > 0.05) than that produced by light alone.

Discussion

In the present study, an initial descriptive approach to understanding how light and 5-HT might functionally interact involved applying light and 5-HT simultaneously and measuring the ensuing phase shifts. The application of 5-HT in combination with light was found to have profound effects on the magnitude of the resulting phase shifts in the ocular rhythm. These results demonstrate that the two phase shifting pathways converge and interact, and illustrate that a putative efferent transmitter can have a major impact on light-induced phase shifts.

Furthermore, the dose response curve (Fig. 2) demonstrates that 5-HT reduced the magnitude of light-induced phase delays in a dose dependent manner. This dose dependency occurred over a range of doses comparable to those previously reported to exert receptor mediated effects in Aplysia (Branelli et al. 1976; Drummond et al. 1980; Corrent and Eskin 1982) and is consistent with a physiological role for 5-HT receptors in this system. In a number of cases in Aplysia, the effects of serotonin are mediated by activation of adenylate cyclase and the resultant increase in cAMP (Drummond et al. 1980; Eskin et al. 1982; Eskin and Takahashi 1983; Levitan and Levitan 1988). This is likely to be the mechanism operating in the present case as recent data indicate that an activator of adenylate cyclase (forskolin, 2 × 10⁻⁶ M) mimicked the effects of 5-HT on light-induced phase delays (Colwell, unpublished).

Light-induced phase advances and delays were differentially affected by the application of 5-HT. The application of 5-HT inhibited the light-induced phase delays but enhanced light-induced phase advances. Moreover, pretreatment with 5-HT enhanced light-induced phase advances yet had no significant effect on the magnitude of light-induced phase delays. This type of phase dependency in the pharmacology underlying light-induced phase shifts of a circadian pacemaker has been previously observed in the golden hamster. Ralph and Menaker (1985) reported that GABA antagonists blocked light-induced phase delays in the circadian rhythm in wheel-running activity but had no effect on the magnitude of phase advances. Together, these results illustrate how a regulatory path which results in the release of a transmitter could have phase dependent effects on the magnitude of light-induced phase shifts.

Finally, the magnitude of the phase shifts due to the interaction between light and 5-HT was dependent upon the sequence of the two treatments. In the late subjective night, 5-HT followed by light resulted in phase shifts which were significantly larger than those produced by the simultaneous application of the two treatments. This result is particularly interesting because it clearly demonstrates that pretreatment with 5-HT can enhance the subsequent response of the circadian system to light. This modulation occurred despite the fact that 5-HT treatment alone (at the same phase) produced no phase shift of the circadian oscillation. Thus, these data are free from some of the interpretational difficulties that arise at the phases when both light and 5-HT treatments produce phase shifts in the ocular rhythm. These data suggest that 5-HT can act to modulate the magnitude of light-induced phase shifts by acting on the light input pathway. Future experiments will focus on the mechanisms which underlie this impact of 5-HT on light-induced phase shifts.

Studies of the interactions between light and 5-HT in the isolated molluscan eye may lead to a new understanding of the functional role of 5-HT in Aplysia's ocular pacemaker system. There is some evidence that 5-HT is a neurotransmitter used by efferent fibers which run from the cerebral ganglion to the eye in Aplysia. Although little is known about the functional role of this serotonergic innervation, one possibility is that 5-HT acts on the isolated eye as an output of an entrainment pathway functionally distinct from light. Another possibility, which was first suggested by Prichard and Lickey (1981), is that 5-HT acts to modulate the light entrainment pathway of the ocular pacemaker. The study presented in this paper is consistent with the later suggestion. These two proposed functions are not mutually exclusive and will need to be specifically examined in experiments in which, for example, recordings are made from the efferent fibers in vivo.

Neurotransmitter systems with properties similar to those described here for 5-HT have been found in other circadian systems. This is best exemplified by the effects of neuropeptide Y (NPY) on the circadian system of rodents. NPY immunoreactive fibers project to the suprachiasmatic nucleus (SCN) of the hypothalamus which is the site of circadian pacemaker cells that generate overt circadian rhythms in mammals (Moore et al. 1984; Harrington et al. 1985; Ralph et al. 1990). The microinjection of NPY into the SCN produced phase shifts of the circadian rhythm of locomotor activity which are qualitatively similar to those phase shifts produced in Aplysia by 5-HT alone. Both agents cause phase delays occurring in the early subjective day and phase advances in the late subjective day (Albers et al. 1984). Light, on the other hand, produces phase delays in the early subjective night and phase advances in the late subjective night in all organisms that have been studied. Perhaps, 5-HT can be considered a well studied representative of a class of agents whose phase response curves are 180° out of phase with light. If so the types of interactions that we have observed between light and 5-HT in Aplysia may occur with respect to other neurotransmitters in other circadian systems.

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