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Serotonin Shifts the Phase of the Circadian Rhythm from the *Aplysia* Eye

Abstract. *A putative neurotransmitter, serotonin, may be used to transmit temporal information in the eye of* Aplysia, *because it can shift the phase of the circadian rhythm of spontaneous optic nerve impulses from the eye and the eye contains a significant quantity of serotonin. Serotonin acts either directly on the cell, or cells, containing the circadian pacemaker or on cells electronically coupled to the pacemaker cells.*

Entrainment of circadian pacemakers (CP's) by light-dark (LD) cycles requires processing of the LD information by a photoreceptor and propagation of the information to the pacemaker. Decoding of this information by the pacemaker shifts the phase of the rhythm. The isolated eye of *Aplysia californica* affords an opportunity to study the mechanisms of entrainment. The isolated eye exhibits a circadian rhythm of spontaneous compound action potentials (CAP's) (*1*) which is entrainable by LD cycles in vitro (*2*). Thus a complete entrainment pathway is contained within the eye. Other pathways for entrainment of the eye may exist, because the CP in the eye may be entrained by extraocular photoreceptors (*3*). Also, the circadian pacemaker of one eye may be coupled to the pacemaker in the other eye (*4*).

Some work on the ocular entrainment pathway has been done (*5*). Treatments that inhibited secretion and blocked nerve potentials (tetrodotoxin with high Mg^{2+} and low Ca^{2+} concentrations) did not affect phase-shifting of the CAP rhythm by light pulses. Thus, neither chemical release nor action potentials are required for shifting the rhythm in the eye by light. Treatments which blocked phase-shifting by light (low concentrations of Na^+ and very low concentrations of Ca^{2+}) all caused a reduction of the electroretinogram of the eye by at least 90 percent. This correlation suggests that the translation of light reception by the photoreceptors into a membrane potential change (photoreceptor potential) is an important step in phase-shifting. Further support for the involvement of membrane potential changes comes from the fact that depolarizing stimuli, strophanthidin, and increased concentrations of extracellular K^+ can shift the phase of the rhythm (*6*).

In the course of these entrainment studies a number of putative transmitters were applied to the eye at phase CT 18–24 (*7*). Light pulses significantly advance the rhythm at this phase. None of the transmitter substances produced net phase shifts when applied to the eye at phase CT 18–24. However, a large variation was noted in the effects of serotonin at this phase. We now report that the putative transmitter serotonin shifts the phase of the rhythm of the *Aplysia* eye when it is applied at other phases, and that serotonin acts either directly on the cells containing the CP or on cells electrotonically coupled to the circadian pacemaker cells.

Eyes with optic nerves attached were dissected from *Aplysia* which previously had been entrained to a 12:12 LD cycle. The eyes then were submerged in buffered (Hepes) filtered seawater (BFSW) (15°C) containing penicillin and streptomycin, and were placed in light-tight boxes under constant dark conditions. Optic nerve activity was recorded on a Grass polygraph by means of platinum wire electrodes. Solutions were added and rinsed out of the eyes by polyethylene tubing which was passed into the boxes through light-tight fittings (*8*).

Treatment of the eyes with serotonin ($10^{-5}M$) for 6 hours advanced the circadian rhythm by 3.0 ± 0.4 hours (95 percent confidence interval; $N = 14$) when it was administered at phase CT 05-11 (Fig. 1A); similar treatment administered at phase CT 20-02 delayed the rhythm by 2.5 hours ($N = 2$). In general, treatment with serotonin advanced the rhythm when it was given during the projected day and delayed the rhythm when given during the projected night (Fig. 1B). The transition from delay to advance occurred at approximately projected dawn. Treatment with serotonin produced 4-hour phase shifts of the rhythm in doses as low as $10^{-7}M$ and had no effect on the rhythm in concentrations over $10^{-3}M$. At intermediate concentrations of serotonin (10^{-3} to $10^{-6}M$), 6-hour treatment appeared to saturate the phase-shifting machinery of the eye, because there were no significant differences among the average phase shifts produced by any concentration of serotonin within this range. The failure of serotonin to shift the rhythm at higher concentrations may be due to desensitization of receptors (*9*). Serotonin also decreased the spontaneous CAP activity of the eye at all phases and concentrations tested (2×10^{-3} to $10^{-7}M$).

The effects of serotonin on the spontaneous activity of the eye and the rhythm suggested that this compound might perform a neurotransmitter or neurosecretory function in the eye. Since a prerequisite for demonstrating a transmitter role for serotonin is to show that it is contained in the eye, groups of two to four eyes were assayed for serotonin and other biogenic amines (*10*). The quantities of these substances per eye were 0.25 ± 0.12 ng of serotonin and 0.02 ± 0.01 ng of dopamine ($N = 6$). Since a single eye contains about 5 μg of protein in its retinal cells (*11*) this equals about 50 ng of serotonin per milligram of protein per eye. This is approximately the concentration of serotonin found in the cerebral ganglion of *Aplysia* (*12*) and considerably greater than the concentration found in the serotonin-rich areas of the mammalian brain (*13*). Serotonin and dopamine were also measured in the optic nerve: the concentrations were, per milligram of protein per nerve, 20 ± 11 ng of serotonin and 2 ng of dopamine (14 μg of protein per optic nerve).

The specificity of serotonin in producing the phase shift was examined in several ways. The immediate precursor to serotonin, 5-hydroxytryptophan, did not produce phase shifts (phase CT 05–11, $10^{-5}M$). Dopamine, a putative transmitter found in the eyes, did not produce phase shifts (phase CT 05–11, $10^{-5}M$).

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Also, four inhibitors of serotonin responses were tested for their ability to block the phase-shifting effect of serotonin. Methysergide maleate (Lilly), Cinanserin-HCl (Squibb), *d*-tubocurarine (Sigma), and tryptamine (Sigma) were all ineffective in concentrations from 10^{-5} to $10^{-3}M$ at blocking the effects of serotonin ($5 \times 10^{-7}M$) on the rhythm. None of these drugs, in any of the concentrations used, had any effect on the rhythm when used alone. Bufotenine (*N,N*-dimethylserotonin) appeared to act as an agonist of serotonin, because it advanced the phase of the rhythm (CT 05-11, 3.4 ± 1.0 hour, $N = 4$, $10^{-5}M$). In addition, the effects of bufotenine on the spontaneous neural activity of the eye were very similar to those of serotonin. Several potential blocking agents for specific types of *Aplysia* serotonin receptors remain to be tested. Also, no blocking agents have been found for the membrane conductance decreases produced by serotonin in *Aplysia* (*14*).

It is possible that serotonin shifts the phase of the circadian rhythm in the eye by stimulating other cells to secrete transmitters which in turn act on the cells containing the circadian oscillator. To examine this possibility, we placed *Aplysia* eyes in a solution containing a low concentration of Ca^{2+} (.01 times normal) and a high concentration of Mg^{2+} (4 times normal) before adding serotonin and during treatment with serotonin. This solution, which should block release of transmitter or secretory substances in *Aplysia* (*15*), did not block phase-shifting by serotonin (the phase shift was 2.3 ± 0.9 hours; $N = 5$). It is still possible that serotonin acts on cells that are electronically coupled to the cells containing the circadian oscillator. This possibility cannot be tested because treatments which disrupt electrotonic junctions also produce phase shifts of the rhythm (*5*).

Several lines of evidence suggest that serotonin may act as a neurotransmitter in the eye: serotonin inhibits CAP's and shifts the phase of the circadian rhythm at $10^{-7}M$; the waveform of the circadian rhythm is affected by prior treatment of the eye with serotonin (*5*); bufotenine, an established analog of serotonin, seems to act on the eye in the same way as a serotonin agonist; finally, the eye contains a significant quantity of serotonin.

Biogenic amines have been identified cytochemically in the eye and optic nerve of *Aplysia* (*16*). Fluorescence indicative of dopamine was observed in secondary neurons (nonreceptor cells) but fluorescence due to serotonin was not observed. Amines were further identified at the ultrastructural level in secondary neurons by a nonspecific chromium-staining technique. The conclusion (*16*) that the reaction product was due to catecholamines must be reconsidered in view of the large amount of serotonin that we found in the *Aplysia* eye. While it might appear surprising that the fluorescence techniques (*16*) did not detect serotonin in the eye, even though the eye contains much more serotonin than dopamine, the same techniques did not reliably record serotonin fluorescence in the giant serotonin-containing cells of the cerebral ganglion (*17*).

Our results represent, to our knowledge, the first demonstration of a shift in the phase of a circadian rhythm by a putative neurotransmitter substance (*18*). Serotonin provides a natural marker that could be further exploited in studies of the circadian system within the eye of *Aplysia*. Information transmission by serotonin may originate in the eye or in some other part of the nervous system. Release of transmitter substances was not necessary for shifting the rhythm by light (*5*). Although the involvement of serotonin in a strictly ocular entrainment pathway appears unlikely from our results, there may be parallel entrainment pathways within the eye, one requiring transmitter release (serotonin), the other

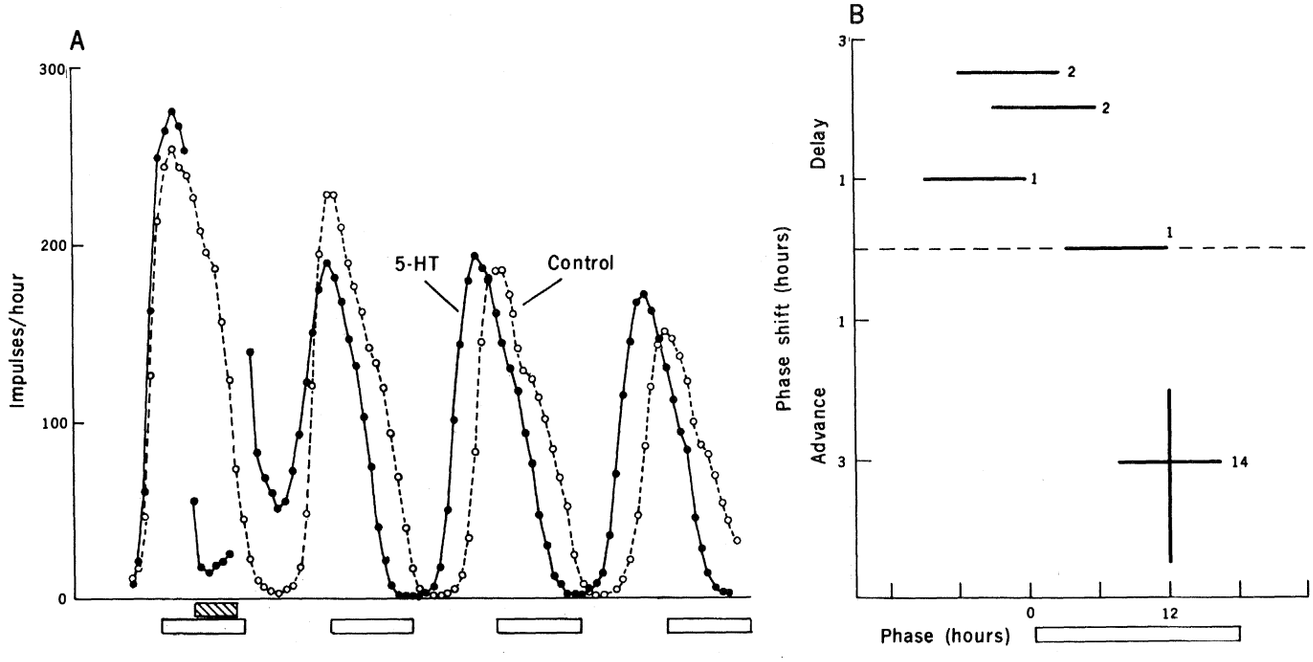

Fig. 1. (A) An advance in the phase of the circadian rhythm from the *Aplysia* eye produced by serotonin (*5-HT*), and (B) a phase response-curve from eyes treated with serotonin for 6 hours. (A) The frequency of spontaneous optic nerve impulses from two isolated eyes of the same animal is plotted against time in constant dark. The open bars at the bottom of the graph represent the projected light portion (12 hours) of the LD cycle to which the animals were entrained before their eyes were removed. The hatched bar spans the time (6 hours) that the experimental eye (closed circles) was exposed to serotonin ($10^{-7}M$). (B) Phase shifts of the rhythm as a function of the time of exposure of the eyes to serotonin ($10^{-5}M$). The horizontal bars are the mean shift at a given phase and span the time of treatment. The numbers to the right of each horizontal bar are the number of experiments at that phase, and the vertical bars span the range of the results at that phase. The open bar at the bottom of the graph spans the projected light portion of the LD cycle to which the animals were entrained prior to dissection of the eyes. Phase shifts were calculated over the second full cycle of the rhythm after the serotonin treatments.

not. Besides its possible participation in ocular entrainment by light, serotonin may be involved in mediating phase-shifting in two other ways: extraocular entrainment of the eye by red light (*3*) and mutual coupling of the circadian pacemakers in the two eyes (*4*). The presence of efferent optic nerve fibers (*2, 19*) and the large amount of serotonin in the optic nerve are consistent with the possibility of an extraocular source of the information on phase-shifting.

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7. Circadian time (CT) 12 is the time of the last offset of light experienced by the intact animals.
8. The details of the experimental procedure and media have been described (*5*). Serotonin and other drugs were dissolved in BFSW with or without ascorbic acid (1 m*M*) which was added to retard oxidation of serotonin. Ascorbic acid had no effect on the circadian rhythm. Bufotenine was dissolved first in dimethyl sulfoxide (DMSO) and added to the tissue in BFSW with a final DMSO concentration of 0.01 percent. Neither this nor a ten times greater concentration of DMSO had any effect on the spontaneous activity or the rhythm from the eye. All solutions containing blocking agents were applied 20 to 30 minutes before treatment with serotonin.
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Medical Technology and Cost Containment: Two Applications of Operations Research

Abstract. *The government proposes "common sense" regulations to help contain rising health costs due to the increasing use of high-technology procedures, such as computerized tomography scanning. Two illustrations are given showing that such ad hoc regulations may have the effect of increasing costs and are certainly far from the optimum obtained by known methods of operations research.*

The delivery of increasingly sophisticated forms of medical care requires the availability of a wide range of medical specialists together with an array of diagnostic facilities and therapeutic equipment operated by skilled personnel. Since the costs in terms of personnel, equipment, and facilities to provide such care are increasing, issues of cost containment in health care have a high priority within the federal government.

The federal government has proposed health planning regulations (*1*) in an effort to contain such increasing costs. Many of these proposed regulations deal with major items of medical equipment, such as computerized tomograph (CT) scanners. Many of the proposed regulations contain specific fixed numerical guidelines that are apparently based on "common sense" rather than applications of the techniques of operations research. For example, for CT scanners, the Department of Health, Education, and Welfare (HEW) would like to see a scanner schedule at least 2500 patients or more per year (that is, 45 minutes per patient for 7^1/$_2$ hours per day for 250 days per year) (*1*). And HEW would like to see a machine load of 4000 patients per year before a second one could be installed (*1*).

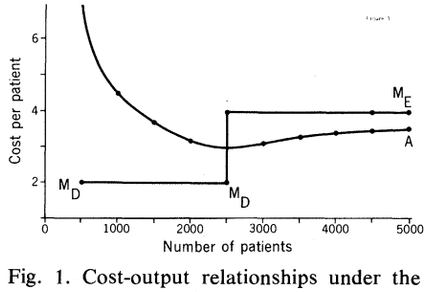

Fig. 1. Cost-output relationships under the government proposal.

As operations researchers and management scientists know, such common-sense approaches can produce far from optimum results. Furthermore, because formal methodologies and quantitative techniques have not yet been presented in conjunction with these numerical guidelines, there is no mechanism to adjust the numerical values (even if correct initially) to changing conditions and circumstances. This is especially unfortunate in high-technology areas in which local variations in patterns of utilization and rapid fluctuations in the cost of equipment and labor are the rule rather than the exception.

We have used operations research methods to show that the specific numerical guidelines in regulating major medical instrumentation can neither minimize the cost per patient nor engender cost-effective decisions. Two illustrations will be given. The first examines a guideline that assumes the cost per patient will be minimized if the number of patients per

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