categorical terms. The results of studies in behavioral pharmacology have forced a reevaluation of the concept of punishment and, at the same time, have yielded a greater understanding of variables contributing to the effects of drugs on behavior. Just as all behaviors controlled by reinforcement are not similarly affected by drugs, neither are all behaviors suppressed by punishment (*9, 12*). The effects of drugs do not depend simply on whether a behavior is reinforced or punished but depend on other more complex and multiple determinants of those behaviors. As demonstrated in this study, prior experience can leave residual effects that, although not manifest in current behavior, can nonetheless significantly influence the behavioral effects of drugs.

JAMES E. BARRETT

Department of Psychology, University of Maryland, College Park 20742

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Neuronal Circadian Rhythm: Phase Shifting by a Protein Synthesis Inhibitor

Abstract. *A potent inhibitor of protein synthesis, anisomycin, was applied (10^{-6}M) in 6-hour pulses at specific phases in the circadian rhythm of endogenous compound action potential (CAP) activity recorded from the eye of* Aplysia *in vitro. The phase of the circadian rhythm was systematically advanced or delayed (up to 15 hours) depending on the specific phase at which the pulse was applied. The resultant phase response curve implicates protein synthesis on the eukaryotic ribosome as a fundamental part of the controlling processes that constitutes the circadian clock.*

Although the basic mechanism for circadian rhythms is thought to be endogenous to cellular biochemical processes, the exact nature of the regulatory system is unknown. Various models for the cellular clock have been proposed (*1*), including a sequential transcription model (*2*) and a membrane model (*3*) that takes into account the results of ionic studies (*4*). The involvement of protein synthesis in circadian rhythms has been tested on several occasions with the use of specific inhibitors. Early results on *Gonyaulax*, a dinoflagellate, were equivocal (*5*), but there was good evidence that cycloheximide increased the period of the rhythm in *Euglena* (*6*). Experiments with puromycin and cycloheximide on the rhythms in *Acetabularia* (*7*), an alga, and *Aplysia* (*8*), a gastropod, show that these inhibitors cause phase-dependent shifts in the rhythms. We now report that low concentrations ($10^{-6}M$) of anisomycin, a potent inhibitor of protein synthesis at the ribosomal level in eukaryotes (*9*), given in 6-hour pulses, either advanced or delayed the phase of the circadian rhythm in compound action potentials (CAP) from the eye of *Aplysia*, depending on the phase of the rhythm at which the inhibitor was applied. This result, together with the studies on other rhythms cited above, is strong evidence for the importance of ribosomal protein synthesis in the cellular regulatory mechanisms that constitute the circadian clock.

The interpretation of the effects of inhibitors on rhythms is dependent on the precision of the phase and the period of the rhythm, and on the degree to which the inhibitor is effective at low concentrations and free of side effects. The *Aplysia* eye rhythm and the inhibitor, anisomycin, meet these requirements exceptionally well. Anisomycin ($10^{-6}M$) applied to *Aplysia* central neurons (*10*) inhibited protein synthesis by 90 percent but did not interfere with RNA synthesis or the physiological function of the neurons. The eye rhythm (*11*) is regular in wave form, period and amplitude when continuously recorded in vitro for weeks (*12*), allowing the accurate determination of the rhythm before and after inhibitor treatment. A persistent change in period or a phase-dependent response to treatment is convincing evidence that the clock mechanism itself is affected (*13*).

Aplysia californica (100 to 300 g), obtained from Pacific Bio-Marine (Venice, Calif.) and kept in Albany in Instant Ocean aquariums in a light-dark period of 13 to 11 hours (LD 13:11) at 15°C were used. Eyes with attached optic nerves were dissected and placed in 125 ml of culture medium, maintained thereafter at 15°C in constant darkness (DD). The optic nerve was drawn into a tubing electrode (*12*) in the culture chamber allowing the CAP to be recorded continuously on a Grass polygraph. The culture medium was similar to one used previously (*12*). It contained 90 percent artificial seawater (ASW) and 10 percent nutrient mixture including MEM (minimum essential medium), essential and nonessential amino acids, vitamins, dextrose, penicillin, and streptomycin (Gibco) (*12*), but no *Aplysia* blood or glutamine. Anisomycin (Pfizer) was dissolved in ASW and added to the culture medium (*p*H 7.8) after the rhythm had been monitored for 1 or 2 days. It was administered in 6-hour pulses (30 eyes tested) or left in continuously for several days. After treatment, the inhibitor solution was removed, the preparation was washed with 250 ml of culture medium, and fresh medium was added under dim red light. Control experiments of changing the culture medium did not perturb the rhythm. Initial studies showed that the incorporation of tritiated leucine into trichloroacetic acid precipitable protein in the eye is inhibited by anisomycin up to 90 percent on days 1 to 3 and 40 percent on day 7 (*14*). Our finding agrees with that of others (*8, 10*), namely, that protein synthesis decreases with time after dissection in *Aplysia* neurons. Protein synthesis is preserved longer by the use of culture medium (*15*) as is the circadian rhythm (*12*) which does not exhibit the pronounced damping observed in seawater alone (*8*).

The CAP activity undergoes smooth changes in frequency in eyes maintained in DD and the persistent rhythm of fre-

quency changes is considered endogenous to the neurons of the *Aplysia* eye (*11*). Under the conditions of this study, cycles in frequency occur with a circadian period of about 26.5 hours, and the high frequency part of the cycle coincides on the first day in DD with what would have been the light period (projected day) of the previous LD cycle (Fig. 1). Typically, the rhythm was monitored in DD for several days to establish the normal phase and period of the eye before anisomycin was applied at a selected phase of the circadian cycle. For example, $10^{-6}M$ anisomycin was applied at the time indicated by the first arrow and withdrawn 6 hours later (second arrow in Fig. 1). When applied at this phase of the circadian cycle, anisomycin caused a large delay in the phase of the rhythm, as compared to the phase of the rhythm in an untreated eye (dashed line in Fig. 1). The experimental phase was compared with the control phase for the first, second, and third cycles after the anisomycin pulse. The rhythm goes through slight variations in period length (transients) (*12, 16*) before becoming stable again by the second or third cycle after the pulse. After the 6-hour pulse of anisomycin, the periods between cycles 1 to 2, 2 to 3, 3 to 4, and 4 to 5 are 26, 28.5, 26.5, and 27 hours compared to control periods of 26.5, 27.5, 27, and 28 hours (measured at the half-maximum frequency increase). This shows that the rhythm was immediately delayed about 12 hours and that, after the inhibitor was removed, the normal periodicity recovered but with a maintained delay with respect to controls.

The delay or advance of the phase depended upon the phase of the rhythm at which the inhibitor was applied (Fig. 2A). Hour 0 corresponds to projected dawn and the time of half-maximum CAP frequency of the free-running rhythm observed in culture medium (Fig. 2B). The average (closed circles) and the range of observed phase shifts are plotted at the midpoint of the 6-hour pulses. The continuous line was drawn by eye to show the trend of phase shifts. Maximum delays were obtained at circadian hour 2 and maximum advances were obtained at hour 5.

Pulses of inhibitor did not perturb the CAP frequency when it was applied or after the inhibitor was washed out. Rather, the inhibitor stabilized the frequency at the rate characteristic for the phase of the rhythm at which the inhibitor was applied (Fig. 1). After the washing, the frequency increased or decreased consistent with the newly adopted phase of the rhythm, suggesting that the inhibitor neither depolarized nor hyperpolarized the membrane potential of the neurons in the eye but stabilized it. This is consistent with studies on central neurons of *Aplysia* (*10*) in which anisomycin (at 18 μM) had no significant effect on membrane properties.

In eight experiments anisomycin was applied continuously for days. The rhythm was abolished at $10^{-6}M$ but the CAP activity proceeded at 50 CAP per hour for 3 days, and the waveform of each CAP was normal. At $10^{-7}M$, two oscillations with long periods occurred before the rhythm damped out. At $10^{-8}M$, the rhythm was near normal but the period was lengthened to 28 hours, suggesting that $10^{-8}M$ is near the threshold for inhibitor effect.

Schwartz *et al.* (*10*) found that protein synthesis in *Aplysia* neurons was not sensitive to streptomycin and other inhibitors that act on the prokaryotic ribosome. *Aplysia* eye rhythm studies are conducted in the presence of streptomycin and penicillin to prevent contamination (*8, 12*) but these substances do not affect the rhythm. Some eukaryotic inhibitors did not block protein synthesis at low concentration (*10*) but Rothman and Strumwasser (*8*) found phase dependent effects on the eye rhythm and inhibition of protein synthesis by 50 percent at 1 to 2 days after dissection. They (*8*) showed a phase response curve for puromycin pulses (6 hours, 125 μg/ml) with maximal delays and advances of 6 hours and a reversal point from delays to advances at hour 0 (projected dawn). The response curve was similar to that for anisomycin but anisomycin pulses caused larger delays (15 hours) at quite low concentrations of inhibitor. In both cases the reversal point from delays to advances occurred at or

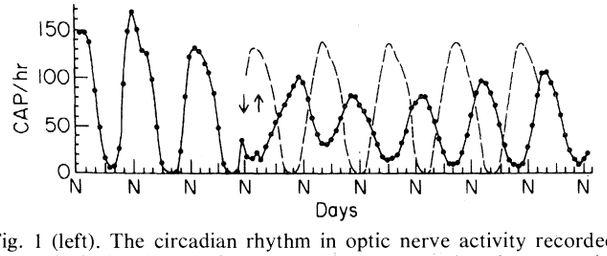
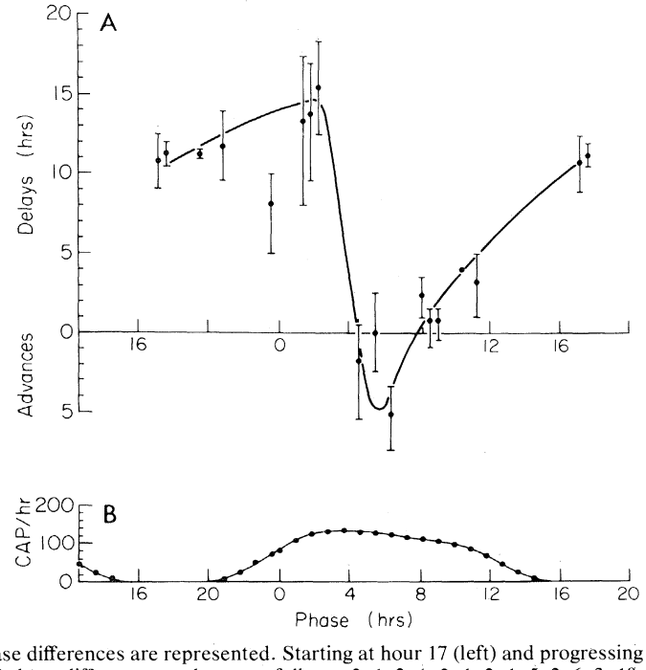


Fig. 1 (left). The circadian rhythm in optic nerve activity recorded from the isolated eye of *Aplysia*. The compound action potential (*CAP*) frequency was plotted every 2 hours for 9 days of continuous recording [abscissae with successive noons (*N*)]. Anisomycin ($10^{-6}M$) was added (first arrow) to the culture medium and removed (second arrow) 6 hours later. The period between the first and second oscillations was 26 hours and the last period after the treatment was 27 hours, showing complete recovery from the treatment. The dashed line shows the expected waveform (from a control eye) if a pulse had not been given. Normal periods increase from 26 to 28 hours over a week or more in culture medium. Fig. 2 (right). Phase response curve for pulse treatment with anisomycin ($10^{-6}M$). (A) The time (hours) of delay or advance of phase is plotted as function of circadian time (hours). The cycle of CAP frequency is shown in (B) for phase reference with hours 16 to 20 repeated. The phase shift for a 6-hour pulse was plotted at the midpoint of the pulse (that is, pulses started at hour 2 and ended at hour 8 were plotted at hour 5). The average phase shift (closed circles) and the range was measured as an advance or delay in phase (ordinate) from the control eyes. In all, 30 eyes and 70 phase differences are represented. Starting at hour 17 (left) and progressing left to right, the eight points for delays represent data from the number of phase differences and eyes as follows: 2, 1; 2, 1; 2, 1; 2, 1; 5, 2; 6, 3; 10, 4; 2, 2. And starting with the first advance at hour 5 the number of differences and eyes are: 12, 4; 4, 2; 6, 2; 4, 2; 3, 1; 3, 1; 1, 1; 6, 2. More than one eye was tested at phases of maximum delays and advance to clearly define the curve. The solid line was drawn to show the trend in phase responses.



near the peak of CAP frequency in the circadian cycle. Interestingly, the reversal point for phase responses to light occurs during the minimum CAP frequency [(*12*), projected night].

In *Acetabularia* (*17*) and *Aplysia* the delays in phase caused by inhibitors of 80*S* protein synthesis occurred at similar times in the circadian cycles (O_2 production at 20°C or CAP activity at 15°C). This common action on such diverse organisms suggests a common mode of action for this class of inhibitors on circadian rhythms generally. The inhibitors (anisomycin, cycloheximide, and puromycin) act on the eukaryotic ribosome (*18*) suggesting that 80*S* protein synthesis is fundamental for the normal functioning of circadian regulatory processes in the cell. There is evidence that synthesis at the ribosome does not itself generate the period of the rhythm in *Acetabularia* (*17*) because the temperature dependence of the period does not match the temperature dependence of the inhibitor effects. Ribosomal protein synthesis as part of the clock is compatible with the membrane model (*3*, *19*) since inhibitors could alter the synthesis or degradation of proteins involved in membrane ionic transport (*19*).

JON W. JACKLET
Department of Biological Sciences and Neurobiology Research Center, University at Albany, Albany, New York 12222

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Selective Destruction of Neurons by a Transmitter Agonist

Abstract. *Microinjection of nanomole amounts of kainic acid, a heterocyclic analog of glutamate, into the cerebellums of adult hamsters and rats causes rapid degeneration of Purkinje, basket, stellate, and Golgi II cells, neurons that receive synaptic input from granule cells, whereas the granule cells themselves are spared. This selectivity is consistent with the evidence that glutamate is the granule cell transmitter and supports the hypothesis that kainic acid exerts its neurotoxic effects through glutamate receptors.*

The selective degeneration of specific groups of neurons with sparing of adjacent, morphologically different groups is characteristic of a number of neurologic disorders. In the cerebellum, granule cells are selectively destroyed by organomercurial poisoning (*1*) and by thiophene (*2*), whereas Purkinje cells degenerate in carcinomatous cerebellar degeneration (*3*) and are destroyed in diphenylhydantoin intoxication (*4*), sodium azide poisioning (*5*), and by an eosinophil extract (*6*). The reasons for this specificity are generally unknown, and it has rarely been possible to relate the selective vulnerability of particular cell groups to their morphologic or chemical properties or functions.

Within the cerebellum, two lines of evidence suggest that glutamate is the granule cell transmitter. First, destruction of the granule cells by virus infection (*7*), radiation (*8*), or genetic mutation (*9*) results in a substantial decline in endogenous glutamate levels without a corresponding decline in the concentrations of other candidate transmitters. Second, destruction of the granule cells is accompanied by a 70 percent decrease in the high-affinity synaptosomal uptake of glutamate (*7*), a process that is thought to be limited to neurons using glutamate as a transmitter.

Systemic administration of monosodium glutamate to immature rodents causes degeneration of neurons in the inner layer of the retina and the hypothalamus. The neurotoxic effects of glutamate appear to be due to its ability to depolarize neurons, since the neurotoxicity of several dicarboxylic and sulfur-containing amino acids structurally related to glutamate is correlated with their neuroexcitatory actions (*10*). A conformationally restricted analog of glutamate, kainic acid, has been shown to be several orders of magnitude more potent than glutamate as a neuronal depolarizer (*11*). Recently, we demonstrated that injection of nanomole amounts of kainate into the striatum produces a selective degeneration of neurons intrinsic to the region (*12*). Therefore, we postulated that if glutamate is the granule cell transmitter and if kainic acid acts on glutamate receptors, the cerebellar neurons that receive granule cell input should have glutamate receptors and should be selectively destroyed by kainate. To test this hypothesis, we injected the cerebellums of rats and hamsters with 2 μg of kainic acid; control animals were similarly injected with 2 μg of α-methylaspartate, a nonneuroexcitatory analog of glutamate (*13*).

Within the immediate vicinity of the injection site there was a small area of necrosis at all periods examined. Thirty minutes after injection, peripheral chromatolysis was already visible in Purkinje cells at the periphery of the injection site, and at 1 hour it was clearly present in basket, stellate, and Golgi II cells as well. Vacuolation was beginning at 1 hour and was much more prominent at 4 and 8 hours, when the area of involvement had a radius of 1.5 to 2 mm. At 24 hours virtually all of the neurons except the granule cells within this radius appeared as pyknotic remnants within large vacuolar spaces (Fig. 1). On electron microscopic examination, peripheral chromatolysis with depolymerization of polyribosomes and cell swelling was present at 30 minutes, and dilatation of the granular endoplasmic reticulum with vacuole formation was extensive by 2 hours (Fig. 2a). These changes pro-