# Extraoptic Phase Shifting of Circadian Locomotor Rhythm in Salamanders

Abstract. *Timing of locomotor rhythm in the slimy salamander,* Plethodon glutinosus, *can be shifted in phase by the environmental light cycle, whether the animals have eyes or not. Rhythmicity persists at least for the first day when animals are transferred to constant conditions, with a period of about 24 hours, and is therefore circadian in nature. An extraoptic photoreceptor site in the brain is suggested.*

Among various environmental variables which can cue an endogenous locomotor rhythm, the overriding influence of light has been demonstrated in a number of animals (*1*), including salamanders (*2*), but the path of reception of this particular cue has received little attention. Several workers have shown that reception of photic information may not be mediated by the obvious photoreceptors. Extraoptic photoreception has been reported in crayfish, insects, frogs, lizards, chickens, ducks, and rats; but only Menaker's recent work with sparrows has implicated such a pathway in entrainment of a circadian rhythm (*3*). This paper reports the existence of an extraoptic photoreceptor in salamanders, which can be used to phase shift a circadian locomotor rhythm.

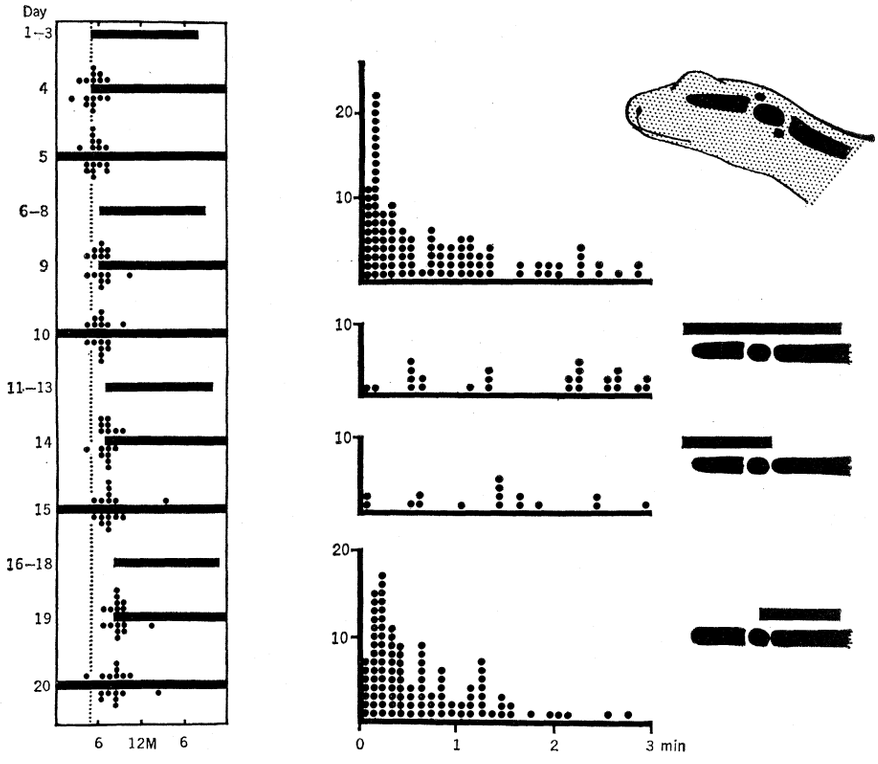


Fig. 1 (left). Persistence of light-shifted locomotor rhythm in *P. glutinosus*. Specimens were subjected to LD 9:15 for 3 days before each of the four 2-day experiments which were recorded; timing of the change from light to dark was shifted from 5:00 p.m. to 6:00, 7:00, and 8:00 p.m., respectively. Other conditions were held constant throughout (15°C, 95 percent relative humidity). The record reads from left to right and top to bottom; the time scale is at the bottom. The black bars represent the periods of complete darkness; the vertical dotted line at 5:00 p.m. is included as a point of reference. Each dot represents the time of initiation of activity of a single specimen; those shown above the black bar have eyes intact, those below it are eyeless. There is a shift in timing of activity and a correlation to the change in timing of light to dark. The rhythm under DD and the amounts of variability in the eyed and eyeless groups after 20 days persists. Fig. 2 (right). Localization of extraoptic photoreceptive site in *P. glutinosus*. The heads of eyeless specimens were selectively covered with opaque paint, and each was then tested for its reaction (in units of time) to the turning on of a fluorescent light. Twenty animals were each tested ten times in each of the four experiments; each dot represents a measurement for a single individual. The diagram at the top shows the relative positions of the various regions of the brain (fore-, mid-, and hindbrain); the solid black bars indicate the approximate position of paint on the head. The response curve obtained from the uncovered heads and that obtained from salamanders with only the forebrain and part of the midbrain exposed are similar, thus suggesting localization of an extraoptic photoreceptive site in this region.

Light entrainment of the locomotor rhythm in the slimy salamander *Plethodon glutinosus* can be accomplished with or without eyes (*4*). Experimental animals respond to changing light-dark (LD) conditions and maintain the previous day's timing for at least a day in constant conditions (DD). Initiation of activity in this nocturnal species is closely correlated with the change from light (fluorescent lamp, 270 lux) to darkness (1 lux), when other cues are held constant (15°C, 95 percent relative humidity). In LD 9:15 (9 hours light, 15 hours dark), with the L to D change being shifted progressively from 5:00 to 6:30 and 8:00 p.m. E.S.T., the correlation coefficient between initiation of locomotor activity and time of change from light to dark is 0.828 [eyed and eyeless groups combined because the two groups are not significantly different ($t = 0.178$)].

To assess the ability to phase shift with light and the persistence of a phase-shifted rhythm in animals both with and without eyes, I tested groups in a separate experiment to compare timing of activity in shifting LD 9:15 and subsequent activity under DD (Fig. 1); other conditions were kept constant. Eight animals were tested in each of the two groups (eyeless, eyes intact). Animals were kept in LD 9:15 for 3½ days before each of four runs in DD; the timing of the change from L to D was shifted from 5:00 to 6:00, 7:00, and 8:00 p.m. on days 1, 6, 11, and 16, respectively (see Fig. 1). Locomotor activity was recorded on only 2 days of each treatment—on the last day that the L to D cue was present and on the following day in constant darkness (DD).

The rhythm can be shifted in phase by changing light cues alone in both the eyed and eyeless groups, and such a phase-shifted rhythm persists for a day in DD in these same groups (Fig. 1). When eyed and eyeless salamanders are allowed to free run (this is, in the absence of external cues) in DD for several weeks, the locomotor rhythm rapidly disappears; apparent arrhythmicity appears typically within a very few days. However, with a recurring though shifting light cue (Fig. 1) the timing is maintained in both groups.

If the blind animals were not receiving light cues (and thus were free-running or becoming arrhythmic), one would expect greater variance in this group after 20 days than in the group with eyes (which could receive cues optically). However, the variances in the two groups are not statistically different at the end of 19 and 20 days ($F = 1.05$ and 1.02, respectively).

Since the eyed and eyeless groups show no statistical differences in timing of activity on any given day, these groups were combined, and two comparisons were made: (i) timing of activity on days when the change from L to D was made at different times, and (ii) timing of activity in LD and subsequent DD. Timing of activity on day 14 is significantly later than that on day 9, and that on day 19 later than that on day 14 ($P = .005$ for both); days 4 and 9 do not differ significantly in timing of activity ($t = 1.627$, $.1 < P < .05$; one-sided test), Thus, there is significant shift in timing of activity when the environmental light regime is shifted. When a comparison is made between timing of activity under LD and subsequent timing of DD (comparing day 4 with 5, 9 to 10, 14 to 15, and 19 to 20), no statistical differences are noted, an indication that the period of the rhythm on the 1st day in DD is not statistically different from 24 hours.

Localization of this extraoptic site was attempted by selectively painting over certain parts of the heads of eyeless specimens and recording the reaction time to the turning on of a fluorescent light (*5*). When the head was unpainted, reaction time was swift (Fig. 2). If the head was completely covered or only the hindbrain was left uncovered, reaction time was considerably slower. If, however, the forebrain and midbrain alone were left uncovered, reaction was swift; and the reaction curve is similar to that obtained when the head was completely uncovered. Attempts to discriminate smaller regions of the head were unsuccessful with this technique. This suggests that the region of the forebrain or midbrain (or both) is photosensitive. Anatomical details support these findings. The brain lies beneath a translucent, cartilaginous skull. Iridophores (a second layer of pigment cells overlying the melanophores) are virtually lacking in the skin over the brain, although they are abundant on most other parts of the body. Furthermore, the distribution of pigment cells on the surface of the brain itself is asymmetric; they are abundant on the hindbrain and most of the midbrain, but completely absent on the forebrain and pineal body. The possibility that the cut ends of the optic nerves perceive the light is rejected, since covering of the top of the head (leaving the optic nerves exposed) eliminated response of the eyeless salamanders (Fig. 2) (*2*).

These data show that phase shifts of the locomotor rhythm can be accomplished in salamanders with or without eyes, and that an extraoptic photoreceptive site appears to be located in the anterior part of the brain. There is still no evidence that the extraoptic photoreceptors in both cases are one and the same, because it has not been possible to block the extraoptic site in the brain for periods longer than a few hours. The location and functional significance of an extraoptic photoreceptor (or photoreceptors) in vertebrates is not at all clear. Research in this area is complex because of the lack of criteria for distinguishing among direct effects on the photoreceptive process, on the behavior being assayed (in this case, locomotor activity), and on the "clock" itself.

There is no experimental evidence that the vertebrate eye is involved in the pathway by which light entrains or phase shifts endogenous rhythms, except in mammals. Possibly extraoptic and optic receptors act in concert under natural conditions; but if so, it is difficult to understand why the extraoptic site alone, as shown in my experiments with salamanders, can synchronize the rhythm with the light cycle seemingly as well as the extraoptic and optic sites can together, at the light intensity tested (Fig. 1) (*2*). Although there are important differences in response to light between eyed and eyeless sparrows, the similarities are more striking than the differences (*6*). In lower vertebrates and birds, it is possible that the eyes are primarily involved in pattern vision and that an extraoptic site mediates light cues for responses in which simple light-on, light-off information is required—phenomena such as thermoregulation, endogenous rhythms, photoperiodic responses, and possibly certain types of orientation (*7*). Mammals, however, react differently. Blinded mice behave as if they were in DD (*8*). The circadian rhythm in the amount of serotonin in the pineals of 12-day-old blinded rats can be influenced by changes in the light cycle, but this response is lost when the rats reach 27 days of age; hooding of the younger rats completely abolishes this response (*9*).

In lower vertebrates, some part of the pineal complex, including the frontal organ (or pineal end vesicle), the parietal organ (in lizards), and the pineal body—as well as the diencephalic roof and the optic tectum—are variously involved in photoreception (*10*). In birds the diencephalic roof or optic tectum may prove to be a photoreceptor since the pineal does not seem to mediate light cues (*11*). In all of these, some part of this complex is anatomically situated in such a way that it could receive photic information. Mammals, however, lack an end organ, and both the pineal body and diencephalic roof are buried in the center of the brain beneath the cerebral hemispheres (*12*). Such a position might preclude sensing of light cues directly by these structures, although the pineal is involved in responses to environmental light perceived optically (*13*). Even though small amounts of light can penetrate to the center of the brains of various mammals (*14*), it has not been demonstrated that such light can trigger physiological or behavioral responses.

These data suggest that light information received for pattern vision, and that received for various physiological responses, are anatomically separate in terms of the sites that receive these photic cues. From considerations of evolution, mammals represent a situation in which both functions, in adults at least, are assumed by the eyes. Such a supposition is speculative but is supported by the data at hand and, I think, provides a useful working hypothesis.

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### References and Notes

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4. Activity was measured by placing animals individually in petri dishes on platforms resting on knife-blade edges. Rocking of platforms would activate microswitches (sensitive to 2.5 g) which were connected to multievent, continuous recorders. All experiments were performed in a Hotpack environmental chamber at Ann Arbor, Michigan; for other details see (*2*). Single units of activity (equal to tripping the microswitches once) were often recorded throughout the day. Thus, it was necessary to establish some criterion for "initiation of activity." This was set at three units per hour; examination of many experiments showed that this criterion in most cases signaled the initiation of an animal's sustained nocturnal locomotor activity. Experimental animals were obtained by H. G. M. Jopson near Bridgewater, Virginia. They were kept in total darkness for 3 days after collection. Then half had their eyes removed, and both groups were placed in petri dishes (day 1). Eyes were removed with scissors after protruding them with slight pressure beneath the head; anesthetics or cold treatment were not used for immobilization since this treatment disturbed the rhythm. All animals were autopsied after experimentation to ascertain that retinas had been completely removed. Temperature of animals did not differ between light-on and light-off conditions at the level of the organisms in any of the experiments, as determined with a thermometer sensitive to 0.1°C.
5. Pactra enamel (black), a fast-drying, opaque paint was used. After the paint dried, the overhead fluorescent lights were turned on, and the time (within 3 minutes) it took for the animals to move was recorded. In most cases, this involved actual movement of the entire animal from one part of the dish to another; in a few instances it meant twitching of the tail or movement of a single limb. All of these were considered to be movement for the purpose of this experiment. To reduce movements due to the endogenous rhythm, all experiments were performed on freshly caught animals from 10:00 a.m. to 3:00 p.m., well before the typical periods of maximum movement at nightfall. See (*4*).
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# Human Oocytes: Maturation in Chemically Defined Media

Abstract. *Human oocytes from ovarian follicles resume meiosis in F10, a defined medium, in numbers comparable to that obtained in medium containing serum. Several simple Krebs-Ringer media also support maturation, which suggests a similarity of nutritional requirements between human and mouse oocytes. Fewer oocytes reach metaphase II when the investing follicular cells are removed prior to culture.*

Human oocytes, when released from ovarian follicles and cultured in medium supplemented with serum, resume meiosis and progress from the germinal vesicle stage through the first meiotic metaphase (metaphase I) to metaphase II with first polar body formation (*1*). This maturation normally occurs in vivo just before ovulation. While oocytes of other mammals will also mature in medium with serum, thus far the only one whose oocytes have been matured in chemically defined media is the house mouse (*2*). In this species, pyruvate or oxaloacetate in a Krebs-Ringer salt solution with bovine serum albumin supports maturation, which suggests the operation of certain metabolic pathways in the oocyte. Mouse follicular cells, when included in the culture system, utilize other energy sources to support maturation, probably by liberating pyruvate into the culture medium (*3*). This report, based on a study of 426 oocytes, describes the first successful use of chemically defined media to support maturation of human oocytes and assesses the need for the presence of the follicular cells (cumulus) during maturation.

In comparing the ability of different media to support maturation, two difficulties were encountered—(i) the number of oocytes obtained by puncturing all visible follicles ranged from 0 to 66 per ovary, with a mean of 10, from 90 patients; (ii) the patients for elective gynecologic surgery varied in age, race, stage of menstrual cycle, and pelvic pathology. Therefore, oocytes from each patient were randomly divided into experimental and control groups under paraffin oil in the collection medium (the medium with the fewest components in each experiment). The control oocytes were cultured in a medium that in our hands had previously supported maturation. In the experiments in which cumulus cells were detached from the oocyte, all cells surrounding oocytes of the experimental group were removed by sucking the oocyte in and out of a drawn Pasteur pipette. Each group of oocytes was washed twice in the appropriate culture medium and finally transferred to microdroplets (two to six per droplet) of this medium (*p*H 7.0) under paraffin oil (*2, 4, 5*).

After 43 to 47 hours at 37°C, oocytes were taken from culture and the cumulus removed from those not previously so treated. Oocytes were then fixed, stained, and examined for the nuclear stage attained (*2*). In only one instance was a polar body not identified when a metaphase II group was

Table 1. Comparison of the number of human oocytes maturing in defined and undefined media. Defined medium, F10 with 4 mg of bovine serum albumin per milliliter; undefined medium, 199 with 15 percent fetal calf serum.

| Patient | Medium | Number of oocytes cultured | Number of oocytes maturing at 43 to 47 hours | | |
|---|---|---|---|---|---|
| | | | Metaphase I | Metaphase II | Total (I and II) |
| 1 | 199 | 19 | 1 | 9 | 10 |
| | F10 | 13 | 0 | 9 | 9 |
| 2 | 199 | 9 | 0 | 6 | 6 |
| | F10 | 9 | 1 | 3 | 4 |
| 3 | 199 | 17 | 3 | 3 | 6 |
| | F10 | 11 | 3 | 6 | 9 |
| 4 | 199 | 4 | 1 | 2 | 3 |
| | F10 | 5 | 1 | 1 | 2 |
| Total | 199 | 49 | 5 (10.2%) | 20 (40.8%) | 25 (51.0%) |
| | F10 | 38 | 5 (13.2%) | 19 (50.0%) | 24 (63.2%) |
| Probability [$\chi^2(1)$] | | | >.75 | >.25 | >.25 |