cells can be induced by azaC (*16*). Further evidence of a role for DNA methylation in the regulation of exogenous viral genes in eukaryotic cells is provided by studies with adenovirus type 12 (*17*) and herpes saimirii (*18*), in which correlations between methylation and gene expression have been observed. The system we have described should allow a specific and quantifiable assessment of the role of DNA methylation in the regulation of gene expression in eukaryotic cells.

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Transplantation of the Cockroach Circadian Pacemaker

Abstract. *Surgical removal of the optic lobes of the cockroach* Leucophaea maderae *followed by transplantation of the optic lobes from another individual led to a restoration of the circadian activity rhythm in 4 to 8 weeks. The free-running period of the restored rhythm was determined by the period of the donor rhythm before surgery. The results suggest that the transplanted optic lobe contains a circadian clock that regenerates those neural connections with the host brain that are necessary to drive the circadian rhythm of activity.*

A generally accepted criterion for the localization of a circadian pacemaker that controls a specific behavioral rhythm is to demonstrate that transplantation of the putative pacemaker tissue from one animal to another (in which the tissue has been removed) both restores a free-running rhythm and confers either the phase or the period of the donor's rhythm on the rhythm of the host. This criterion has been successfully met in three organisms: the silk moth (*1*), the fruit fly (*2*), and the sparrow (*3*). In each of these cases, the transplanted tissue appears to control rhythmicity by release of a hormone, and neural connections between the pacemaker tissue and the nervous system of the host are not required.

In the cockroach *Leucophaea maderae*, there is substantial evidence to suggest that the circadian pacemaker driving the rhythm of locomotor activity is composed of two bilaterally paired and mutually coupled oscillators—one in each optic lobe of the protocerebrum—that are independently able to drive rhythmicity (*4–6*). However, the ability of the optic lobe to sustain a rhythm of activity depends on intact neural connections between optic lobe and the midbrain (*4, 5*). This requirement seemed to preclude critical transplantation experiments. I have discovered, however, that although bilateral section of the optic tracts invariably abolishes the free-running rhythm of locomotor activity, if the optic lobes are left in situ, the rhythm consistently reappears in 3 to 5 weeks, with a free-running period near that of the rhythm before surgery (*7*). The return of rhythmicity likely depends on regeneration of neural connections between the optic lobe and the midbrain since (i) if the optic lobes are removed from the animal, arrhythmicity persists indefinitely (*4, 7, 8*); (ii) histological (*7, 9*), electrophysiological (*7*), and behavioral (*9*) data show that regeneration between neurons of the optic lobe and midbrain does occur; and (iii) the time course of regeneration is similar to that of the return of rhythmicity (*7*).

These results prompted an effort to transplant the optic lobes from one animal into another whose own optic lobes had been removed. In these experiments, adult males that had been raised from birth in LD 11:11 or LD 13:13 were used (*10*). The periods (τ) of the free-running activity rhythms of animals that have been reared in these two conditions are substantially different (*11*). The average τ in constant darkness for males raised in LD 11:11 is 22.7 ± 0.27 (standard deviation) hours (N = 21), and for males raised in LD 13:13 is 24.2 ± 0.26 hours (N = 15). A period difference of more than 1 hour is maintained between the two groups for at least 5 months after the animals are transferred to constant darkness (*11*) and probably persists throughout the life of the cockroach (*12*).

Two separate series of transplantation experiments were performed with a total of 18 animals (nine from each light cycle). Individuals were placed in activity monitors in constant darkness for approximately 4 weeks to determine the period of the free-running activity (*13*). Optic lobes were then exchanged between individuals of the two groups, and the animals were returned to the activity monitors (*14*). Of the 18 animals, 12 survived for 40 or more days after surgery.

Activity was invariably disrupted and apparently aperiodic for several weeks after transplantation; however, in 10 of the 12 animals that survived surgery, a clear circadian rhythm of activity reappeared between 26 and 56 days postoperatively (*15*) (Fig. 1). The remaining two animals were a pair between which the optic lobes had been exchanged. Activity patterns of these two animals, recorded for 75 days after surgery, showed no convincing evidence of periodicity.

In every case in which a rhythm was reestablished, the free-running period was near the period of donor animal's rhythm before transplantation of the optic lobes (Fig. 2). In most cases, there was either an increase or decrease of more than 1 hour in the period of the host rhythm—a major change in τ for adult *Leucophaea* in which the free-running period is normally stable (*11*). It is substantially larger than any period changes occurring spontaneously (*11, 16*), as a result of aging or aftereffects of entrain-

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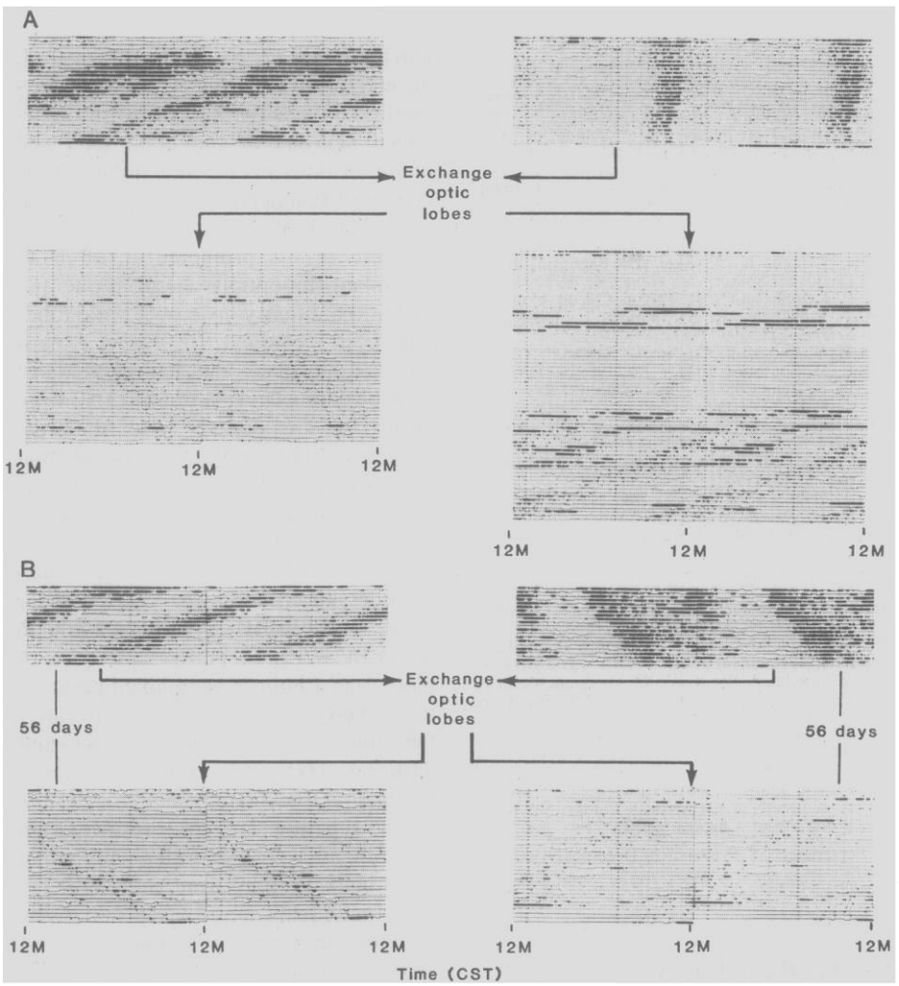
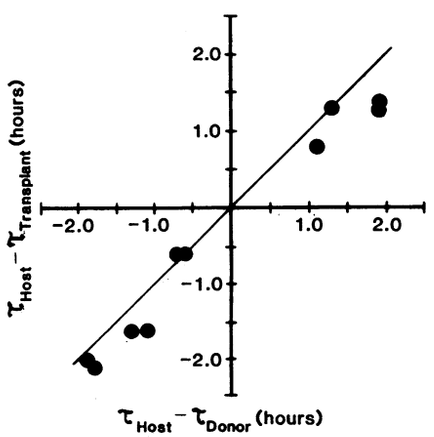


Fig. 1. Records of activity of two pairs of animals, free running in constant darkness, showing effects of optic lobe transplantation. Data for successive days were placed below each other, and each record has been duplicated to provide a 48-hour time base to aid in visual inspection of the data. Animals to the left in each figure were raised in LD 11:11 and animals to the right were raised in LD 13:13. (A) Continuous records. (B) The activity between the time of surgery and the reappearance of a clear rhythm has been omitted. For 4 to 8 weeks after optic lobe transplantation, the activity is sparse and apparently aperiodic. A clear rhythm of activity subsequently reappeared in all four animals, with the period of the rhythms near the period of the donor rhythm before surgery.



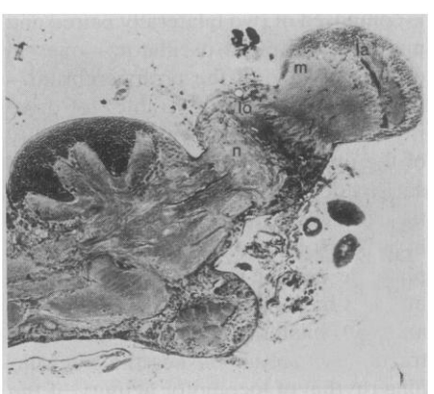


Fig. 2 (left). Change in the period of the activity rhythms of host animals ($\tau_{Host} - \tau_{Transplant}$) after optic lobe transplantation, plotted as a function of the difference in τ between host and donor prior to surgery. The diagonal line is the predicted relationship if τ of the regenerated rhythm is equal to τ of the donor's rhythm. The data conform to prediction remarkably well, even in cases where an increase or decrease in τ of nearly 2 hours was expected. Fig. 3 (right). Section through brain and transplanted optic lobe 73 days after surgery. Portions of the lamina (*la*), medulla (*m*), and lobula (*lo*) of the lobe are visible. Typically, transplanted lobes became attached to the lateral neuropil of the brain (*n*), which bulges outward after optic tract section. Although it is not clear in this section, fiber tracts could usually be found connecting the lobe to the midbrain.

ment of adults (*11*), or as a consequence of surgical interference in the nervous system (*5*, *17*).

For eight of the ten successful transplants and for the two animals that were not clearly rhythmic, the activity recording was terminated before the animal's death, and the brain with the optic lobes was removed and prepared for histological examination (*18*). In five of the eight animals in which rhythmicity returned after surgery, both of the transplanted lobes appeared healthy and had reestablished structural connections with the midbrain of the host. Typically, there was some distortion in the shape of the lobe, as well as the midbrain; however, all three regions of neuropil in the lobe were clearly recognizable, and there was no evidence of any significant degeneration (Fig. 3). In the remaining three animals, there was clear evidence for regeneration of connections for only one of the lobes, although both lobes appeared relatively normal in histological section. In contrast, in one of the animals in which a rhythm was not reestablished, only one of the transplanted lobes was recognizable, and it appeared to have undergone substantial deterioration. In the other aperiodic animal, neither of the transplanted optic lobes could be identified, although encapsulated masses of tissue were present at the transplantation site.

The above data show unequivocally that transplantation of the optic lobes from one animal into another whose own lobes have been removed both restores rhythmicity in locomotor activity and imposes the period of the donor's rhythm on the regenerated rhythm of the host. The results suggest that in transplanting the optic lobes, a circadian pacemaker is transplanted with its characteristic free-running period and that it subsequently regenerates those neural connections with the host central nervous system that are necessary to drive the activity rhythm. However, the transplantation results are not unequivocal proof for this interpretation; a plausible alternative explanation, for example, is that the optic lobe provides an input to an oscillator in the midbrain that is both necessary to sustain the oscillation and has a major impact on its period. Nevertheless, the view that the optic lobe contains a self-sustaining oscillator that functions as the circadian pacemaker for the locomotor activity rhythm seems the more likely explanation. Localized cooling of the optic lobe produces a phase shift in the activity rhythm, whereas cooling the midbrain has no effect (*6*). Thus, the optic lobe appears to dictate

both the phase and the period of the free-running rhythm. In conjunction, these results provide compelling support for the proposition that the circadian clock in the cockroach is in the optic lobes.

It is remarkable that the transplanted protocerebral tissue survives, undergoes functional regeneration, and conserves the period of the free-running oscillation so reliably. A question that arises is whether or not the pacemaker continues its motion between the time of its removal and the return of overt rhythmicity in the host. However, there was no clear indication that phase, as well as period, was conserved in the transplanted lobe (*19*). A positive result would have indicated that the oscillation had persisted; a negative result is open to a number of interpretations, some of which do not preclude the possibility that a circadian oscillation persists in the isolated optic lobe.

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10. Colonies of animals were maintained in light-tight boxes at constant temperature. Light-dark cycles (11 hours of light and 11 hours of darkness or 13 hours of light and 13 hours of darkness) were provided by a 4-W fluorescent light.
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13. Activity was monitored in Lucite cages with running wheels that activated event recorders (*5*). In the first experimental series, animals were separated in individual lighttight boxes; in the second experiment, the activity monitors were located in a single incubator. All experiments were run at 25° ± 0.5°C. Free-running periods were estimated by fitting (by eye) a line through activity onsets. With one exception, all period estimates are based on three or more weeks of data.
14. Surgery was performed with CO_2 anesthesia. Two three-sided cuticular flaps were cut in the head capsule which, when raised, exposed the brain. Fine scissors were used to cut the optic nerves and optic tract of each optic lobe. The lobes were removed from the head capsule and placed in physiological saline at room temperature while a second animal was similarly prepared. The lobes of the two animals were then exchanged, placed in close apposition to the midbrain of the host. Care was taken to maintain the orientation and handedness of the transplanted lobes. The cuticle was repositioned and sealed with wax.
15. Deciding the precise day on which the rhythm returns is subjective—the values given represent the extremes of the earliest and latest times a rhythm was judged to have been present after transplantation. The average number of days to return of a clear behavioral rhythm for all animals was 39 ± 8.9 (standard deviation) days.
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17. Unilateral optic lobe ablation or optic tract section consistently causes a slight increase in τ in animals raised in LD 12:12 ($\overline{\Delta\tau}$ = 0.21 ± 0.23 hours, N = 39) (*5*). Comparable small increases in τ resulting from unilateral optic tract section are found in animals raised in LD 11:11 ($\overline{\Delta\tau}$ = 0.27 ± 0.29 hours, N = 6) or in LD 13:13 ($\overline{\Delta\tau}$ = 0.16 ± 0.22, N = 5). The increase in τ after surgical interference of the optic lobe has been interpreted by use of a coupled oscillator model in which the pacemaker of each optic lobe has a period slightly longer than that of the coupled pair (*5, 6*). This may also explain the tendency for τ of the regenerated rhythm after optic lobe transplantation to be slightly longer (on the average by 0.2 ± 0.24 hour) than τ of the donor rhythm, if normally only one of the two lobes is successful in reestablishing the specific neural connections with the midbrain that are necessary to drive rhythmicity.
18. Brains were fixed overnight in an alcohol-formaldehyde solution, dehydrated, cleared, and embedded in paraffin. Serial sections (12 μm) were cut and stained in 1 percent methylene blue.
19. With the onset of activity as a phase reference point, the phase of the free-running rhythm was projected back to the day of surgery. There was no clear correlation between the rhythm phase and the phase of the donor, the phase of the host, or the time of surgery.
20. Supported by grants from the University Research Council and the Natural Sciences Council of Vanderbilt University.

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Ornithine Decarboxylase: Essential in Proliferation but Not Differentiation of Human Promyelocytic Leukemia Cells

Abstract. *The ornithine decarboxylase inhibitor* DL-α-*difluoromethyl ornithine inhibited a proliferation-associated increase in ornithine decarboxylase activity in cultured human promyelocytic leukemia cells, resulting in a marked suppression of cell proliferation and subsequent cell loss. It also inhibited increases in ornithine decarboxylase activity associated with the phorbol ester–induced conversion of promyelocytic HL-60 cells to monocyte-like cells and the retinoic acid–induced conversion to granulocyte-like cells. However, the inhibition of ornithine decarboxylase activity did not prevent cellular differentiation. These results suggest that polyamine biosynthesis has a specific role in cell proliferation rather than in inducing differentiation that is not accompanied by proliferation. The data also demonstrate that cessation of proliferation in HL-60 cells is not necessarily associated with differentiation.*

The decarboxylation of ornithine by ornithine decarboxylase (ODC; E.C. 4.1.1.17) leads to the formation of putrescine and is the first rate-limiting step in polyamine biosynthesis. Marked increases in ODC activity and polyamine biosynthesis accompany the onset of proliferative events in most cell types studied (*1, 2*). In addition, certain differentiative processes, such as responses of target tissues to specific hormonal stimuli (*3*) and induced differentiation of Friend mouse erythroleukemia cells (*4*), have been associated with increased ODC activity. The importance of ODC in cell growth and differentiation was recently demonstrated in studies with DL-α-difluoromethyl ornithine (DFMO), an inhibitor of ODC (*5*). Inhibition of putrescine synthesis by DFMO arrests the growth of mouse L-1210 leukemia cells and rat hepatoma cells in culture (*6*) and leads to eventual death of human small-cell lung carcinoma in vitro (*7*). DFMO completely suppresses the sharp rise in uterine ODC activity accompanying murine embryogenesis, arresting embryonic development (*8*). Also, DFMO suppresses increases in ODC which accompany the maturation of intestinal mucosa and recovery from injury; both cellular processes are inhibited by the block in putrescine biosynthesis (*9*).

Increased ODC activity may thus play an important role in the initiation of both cellular proliferation and differentiation. It has been difficult, however, to evaluate the processes of proliferation and differentiation separately. Hence it has been unclear whether one or both cellular responses depend on polyamine biosynthesis. Recently, a cell system for studying these events separately was described (*10–12*). The human promyelocytic leukemia cell line HL-60 can be chemically induced to undergo terminal differentiation, probably without accompanying proliferation.

The cell line, derived from peripheral leukocytes from a woman with acute promyelocytic leukemia, contains predominantly leukemic promyelocytes (*13, 14*). HL-60 cells cease proliferation and "differentiate" morphologically and functionally toward granulocytes after the addition to the culture of such compounds as dimethyl sulfoxide, butyric acid, dimethylformamide (*10*), and retinoic acid (*11*). The cells can also be induced to differentiate along an alternate pathway, apparently toward monocytes, when treated with certain phorbol esters, especially 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (*12*). Thus, HL-60 cells are remarkable in their capacity to differentiate terminally despite

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