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Brain Control of Embryonic Circadian Rhythms in the Silkmoth *Antheraea pernyi*

Ivo Sauman* and Steven M. Reppert†
*Institute of Entomology
Czech Academy of Sciences
370 05 Ceske Budejovice
Czech Republic
†Laboratory of Developmental Chronobiology
Pediatric Service
Massachusetts General Hospital
and Harvard Medical School
Boston, Massachusetts 02114

Summary

The clock protein PER is necessary for circadian control of egg-hatching behavior in the silkmoth *Antheraea pernyi*. Since the brain and midgut of the silkmoth embryo contain PER-positive cells, we examined the circadian clock potential of these embryonic tissues. Transplantation experiments indicate that the circadian clock controlling egg-hatching behavior resides in brain, and that a humoral factor mediates this circadian regulation. We also used ligation experiments on first instar larvae to show that the circadian control of PER movement into the nuclei of midgut epithelial cells is dependent on an intact (connected) brain. These results implicate a novel brain factor in the circadian regulation of egg-hatching behavior and provide further evidence for differing mechanisms of PER control among species.

Introduction

Egg hatching is a profoundly important event in the life history of lepidopterans. In the silkmoth *Antheraea pernyi*, a behavioral program is initiated in the early morning hours on the day of hatching whereby the pharate larva begins to gnaw its way out of the egg (Riddiford and Johnson, 1971; Sauman et al., 1996). It takes about 90 min of continuous chewing to make a hole in the chorion large enough for the animal to escape. Proper initiation of this behavioral program is critical because it frees the larva from the constraints of life in the egg to fulfilling its biological destiny in the outside world.

Egg hatching is the earliest recognized behavior under circadian control in the silkmoth (Sauman et al., 1996). At 23°C and under a lighting cycle of 12 hr of light and 12 hr of darkness (12L:12D), pharate larvae hatch within a 3-hr interval (gate) at the dark-to-light transition on days 10–12 of embryogenesis. The hatching rhythm is circadian because it persists in constant darkness (DD). Furthermore, the rhythm can be entrained by light by midembryogenesis. Importantly, the time of appearance of photic entrainment by the silkmoth embryo coincides with the appearance of the protein products of two clock genes, *period* (*per*) and *timeless* (*tim*), in eight cells in brain (Sauman et al., 1996).

Based largely on studies in *Drosophila melanogaster*, *per* and *tim* have been shown to be essential elements of a circadian clock that controls behavioral rhythms (reviewed by Reppert and Sauman, 1995; Dunlap, 1996; Hall, 1996). The mRNA and protein products of both genes oscillate, and the cycling of each is dependent on PER–TIM association and nuclear entry. Thus, *per* and *tim* form interdependent autoregulatory transcriptional loops that are essential components of a *Drosophila* circadian clock. Examination of PER-containing cells in adult *A. pernyi* brain shows that PER is restricted to eight neurosecretory cells. These cells appear to be circadian clock cells because PER protein and *per* RNA are colocalized and their levels oscillate in these cells (Sauman and Reppert, 1996b). However, PER does not cycle in and out of the nucleus in silkmoth brain, as it does in *Drosophila*. Thus, the molecular details of PER dynamics differ substantially between the fruit fly and silkmoth.

We previously showed that PER is necessary for circadian control of egg-hatching behavior in *A. pernyi* (Sauman et al., 1996). *per* antisense oligodeoxynucleotide treatment of pharate larvae on the day before hatching abolishes the circadian gate of egg-hatching behavior. Moreover, *per* antisense treatment causes a dramatic decrease in PER immunoreactivity in newly hatched larvae. Thus, PER-positive cells in the embryo are likely sites of a circadian clock controlling egg-hatching behavior.

Although PER and TIM are expressed in eight cells in embryonic brain, we have been unable to detect a circadian oscillation in protein levels at this developmental stage (Sauman et al., 1996). A robust circadian oscillation of PER immunoreactivity, however, is present in the nuclei of midgut epithelium from day 7 of embryogenesis (Sauman et al., 1996). Since PER-positive cells in brain or midgut could be the site of a circadian clock that controls egg-hatching behavior, we examined the circadian clock potential of each tissue. Transplantation experiments indicate that the circadian clock controlling egg-hatching behavior resides in brain and that a humoral brain factor mediates this circadian control. We also used ligation experiments to show that the circadian control of PER movement into midgut nuclei is dependent on a connected brain. These results (1) implicate a novel brain factor in the circadian regulation of egg-hatching behavior and (2) provide further evidence for differing mechanisms of PER control among species.

Results

Diurnal Egg-Hatching Behavior Is Widespread among Silkmoths

We evaluated the timing of egg hatching in a variety of silkmoths to determine the generality of the circadian control of this behavior. Egg-hatching behavior was examined in four genera (*Actias*, *Antheraea*, *Hyalophora*, and *Samia*) within the family *Saturniidae*. Two species other than *A. pernyi* were examined for the genus *Antheraea*. Of particular interest was *Antheraea yamamai*

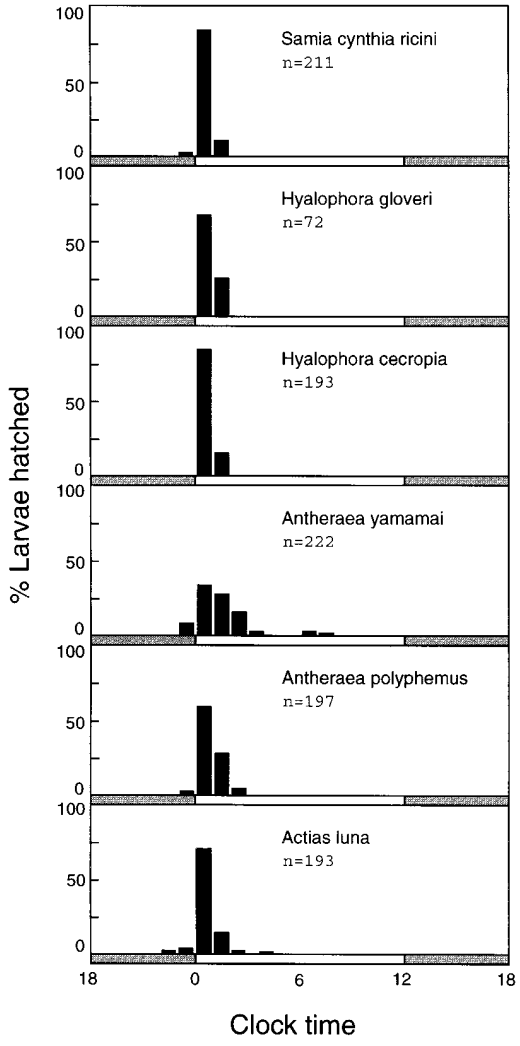

Figure 1. Diurnal Gate of Egg-Hatching Behavior Is Widespread among Silkmoths

Temporal profiles of hatching times were studied under 12L:12D. The hatching data for each species are grouped according to the clock time at which the larvae hatched. The vertical axes indicate the percentage of total hatching events in each 1 hr interval. The horizontal bars depict the lighting cycle; open is light phase and closed is dark phase.

because, unlike the other species studied, *A. yamamai* has an obligatory egg diapause (Suzuki et al., 1990). The embryos of *A. yamamai* develop normally to pharate first instar larvae. From that point, the larvae remain in the egg throughout the winter months. Egg diapause is broken in the spring when ambient temperature begins to rise.

In all species examined, there was a precise diurnal gate of egg-hatching behavior in 12L:12D (Figure 1). A similar diurnal gate has also been reported for *Bombyx mori* (Sakamoto and Shimizu, 1994); this insect exhibits egg diapause in which embryonic development is arrested hours after fertilization. Remarkably, the phase of the egg-hatching rhythm relative to the light–dark cycle is the same for all species, with hatching occurring around the dark-to-light transition (Figure 1). This contrasts with the different phases of the adult eclosion gates and flight activity rhythms among the seven species examined. For example, the adult eclosion gate for *A. pernyi* is in the late afternoon, while that for *H. cecropia* is in the morning (Truman and Riddiford, 1970). In most species we examined, the egg-hatching gate anticipated lights-on in the morning, suggesting that the gate in each species is indeed controlled by a circadian clock, as in *A. pernyi*. These results show that circadian control of egg-hatching behavior is common among silkmoths and may be widespread among lepidopterans.

A Brain Factor Controls the Circadian Gate of Egg-Hatching Behavior

Since the behavior of egg hatching involves coordinated mandibular activity (chewing behavior), the embryonic brain cannot be removed without damaging structures necessary for chewing activity. We thus developed a transplantation strategy in which both the brain and midgut remain intact in transplant-recipient animals. A brain or midgut for transplantation was derived from a donor animal, which was exposed to a 12L:12D lighting cycle that was 8 hr out of phase (either advanced or delayed) from the 12L:12D lighting cycle of the recipient animal (Figure 2). In this way, behavior in the recipient animal could "choose" between the circadian time of the host or transplanted tissue. Transplantation was performed during the 4 hr of light that overlapped between the two lighting cycles. The recipient pharate larvae were judged to be at a stage of development in which egg-hatching behavior would occur during the next available gate. After transplantation, eggs were placed in DD and egg hatching was monitored at hourly intervals.

The first experiment was designed so that the transplanted tissue would convey circadian information that would signal an egg-hatching gate 8 hr later than that normally found in unoperated (control) animals (Figure 2, left). The larvae with a midgut transplant hatched during the normal (earlier) circadian gate with no evidence that the transplant was exerting any circadian control. The larvae with a brain transplant also hatched during the earlier circadian gate, but there was evidence that a small number of larvae (11%) used the later gate (dictated by the transplanted brain).

The second experiment was designed so that the transplanted tissue would convey circadian information that would provide an egg-hatching gate 8 hr earlier than that normally found in control animals (Figure 2, right). Once again, the larvae with midgut transplants exhibited a normal circadian gate with no evidence that the transplants advanced the gate. In contrast, most larvae with brain transplants hatched during the earlier (advanced) gate; that is, they exhibited a behavioral gate that correlated nicely with the circadian time of the transplant. Only a small number of animals with brain transplants (6%) hatched during the later (control) gate. This result strongly suggested that the brain is controlling the circadian gate of egg-hatching behavior. However, it was important to rule out the possibility that just the presence of a transplanted brain itself resulted in hatching at the earlier gate (compare lower left and right panels of Figure 2).

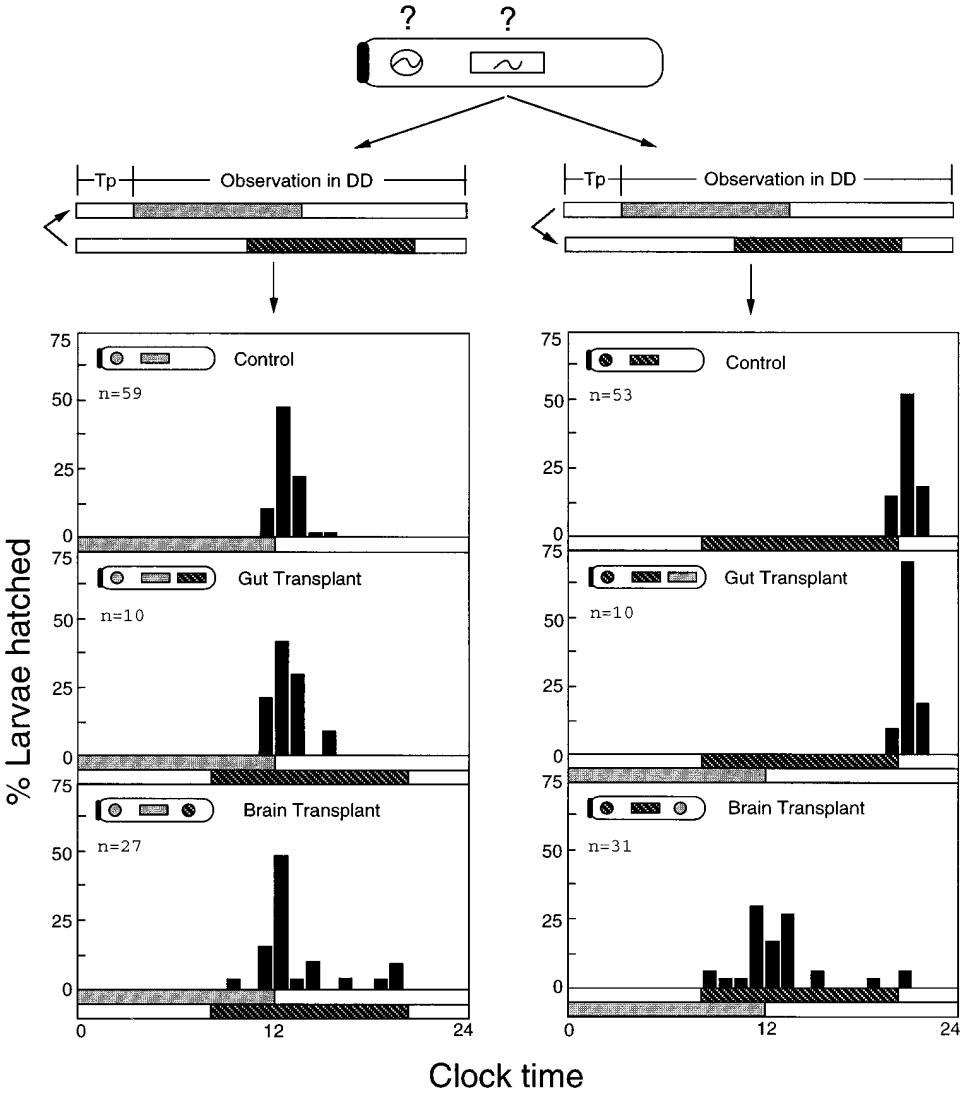

Figure 2. Brain/Midgut Transplantation Experiment

At the top is a diagram of the two potential sites of circadian clocks in pharate larvae, the brain (circle) or midgut (rectangle). This diagram is used in the lower left and right panels to depict the lighting cycle to which the brain (host or transplant) or midgut (host or transplant) was exposed. The left and right lighting schedules below the top diagram show the lighting cycles of donor and recipient animals. The direction of the arrow depicts the transplantation paradigm. Also shown is the overlapping portions of the two lighting cycles in which the transplantation procedure (Tp) was performed. After transplantation, egg-hatching events were observed in constant darkness (DD). Left panels: hatching events for control (upper), midgut transplant (middle), and brain transplant (lower) groups in which the transplanted tissue conveyed circadian information 8 hr later than that found in control animals. Right panels: hatching events for control (upper), midgut transplant (middle), and brain transplant (lower) groups in which the transplanted tissue conveyed circadian information 8 hr earlier than that found in control animals. The horizontal bars depict lighting cycles; open is day (or subjective day) and closed or hatched is night (or subjective night).

To confirm that the circadian time of the transplanted brain was indeed exerting circadian control of egg-hatching behavior, a third experiment was performed. For this experiment, two brain transplant groups were used. The only difference between the two transplant groups was the phase of the light–dark cycle to which the transplanted brain was exposed. For one group, a brain of the same circadian phase as the recipient was transplanted (Figure 3A). For the second group, a brain of a circadian phase that would advance the egg-hatching gate to 8 hr earlier from that found in control animals was transplanted (Figure 3B). The results were unequivocal. The circadian time of the transplanted brain (and not just the presence of a second brain) directed which circadian gate was used (compare Figures 3A and 3B). Thus, the circadian clock regulating egg-hatching behavior resides in brain. The transplanted brain has this influence within 12–18 hr, before neural connections could be established, consistent with a humoral factor from brain mediating circadian control.

An Intact Brain Exerts Circadian Control over the Nuclear Movement of PER in Midgut Epithelium

Since the brain controls the circadian gate of egg hatching, we examined whether the brain also regulates the

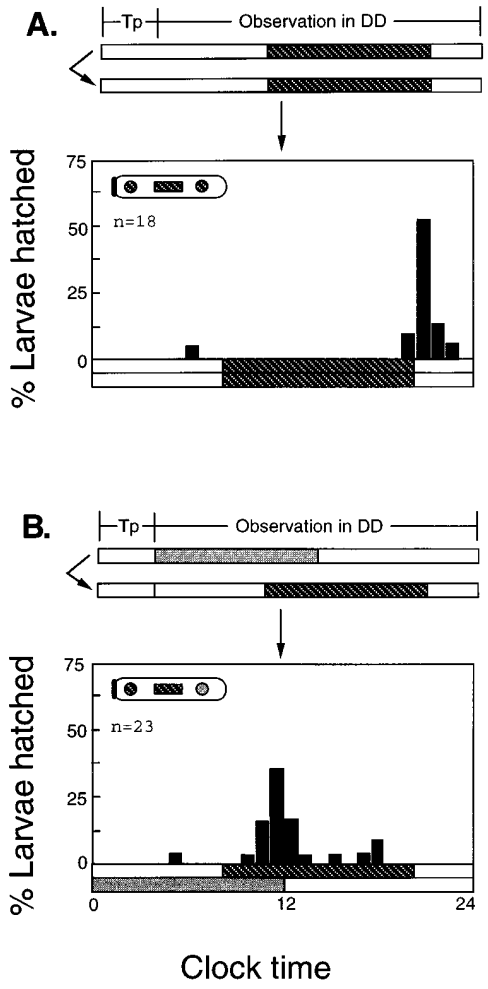

Figure 3. Transplantation Experiment Using Brains of Different Circadian Phase

(A) Upper portion shows lighting cycles of host and transplanted brains; the host and transplant brains conveyed the same circadian phase. The transplantation procedure (Tp) was performed during the first 4 hr of the light period. Lower panel depicts hatching events at hourly intervals.

(B) Upper portion shows lighting cycles of host and transplanted brains; the transplanted brains conveyed circadian information 8 hr earlier than that found in control animals. The time of the transplantation procedure (Tp) is depicted. Lower panel depicts hatching events at hourly intervals.

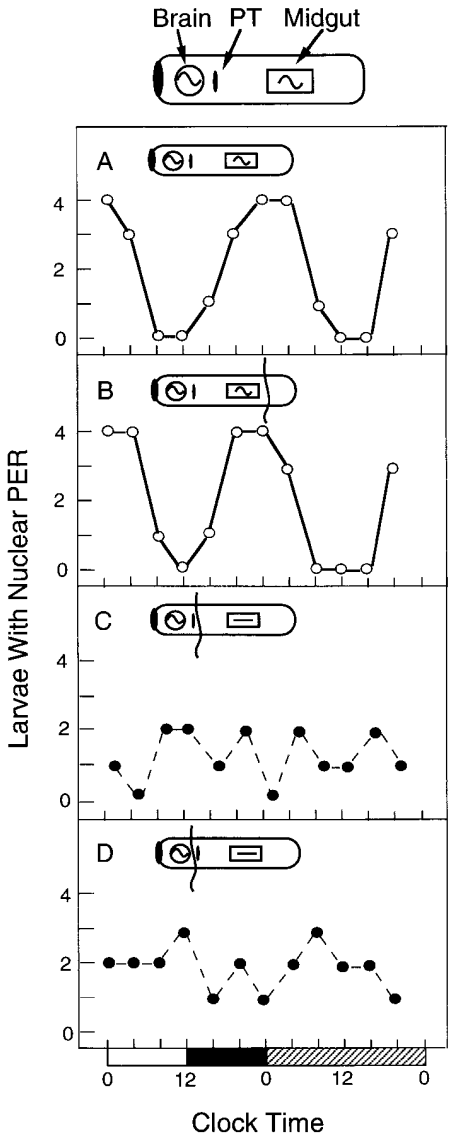

Figure 4. Ligation Experiments of First Instar Larvae

At the top is a cartoon of a first instar larva delineating brain, prothoracic glands (PT), and midgut. This cartoon is used to depict the ligation experiment done for each of the lower panels. The lower panels depicit temporal patterns of nuclear PER in midgut epithelium of control larvae (A), larvae in which distal abdomen was ligated ([B]; ligation control), larvae in which the head and first thoracic segment were ligated (C), and larvae in which only the head was ligated (D). For each panel, four animals were examined at each time point, and data are plotted as the number of animals in each group in which nuclear PER was evident by immunocytochemistry. The horizontal bar depicts the lighting cycle; open is light phase, closed is dark phase, and hatched is subjective day and night.

temporal movement of PER into the nuclei of midgut epithelial cells. This was evaluated in newly hatched first instar larvae. Within the first hour after hatching, a ligature was used to isolate different parts of a larva from each other. After ligation, PER in midgut epithelial cells was monitored by immunocytochemistry at 4 hr intervals for 24 hr in 12L:12D followed by 24 hr in DD.

Unoperated animals exhibited a prominent oscillation in nuclear PER in midgut epithelium in 12L:12D, with maximal levels occurring during the latter half of the dark period (Figure 4A). The rhythm persisted unperturbed for one day in DD. A ligation that isolated the distal abdomen from the rest of the animal (ligation control) did not alter the amplitude or waveform of the nuclear PER rhythm in midgut epithelium in either 12L:12D or DD (Figure 4B). When the head and first thoracic segment (which included both brain and prothoracic glands) were separated from the rest of the body by ligation, there was an immediate dysregulation of PER movement in the nuclei of midgut epithelial cells (Figure 4C). At most times examined, some larvae showed PER staining in midgut epithelial nuclei. In the animals in which PER was either weakly detected or not detectable in the

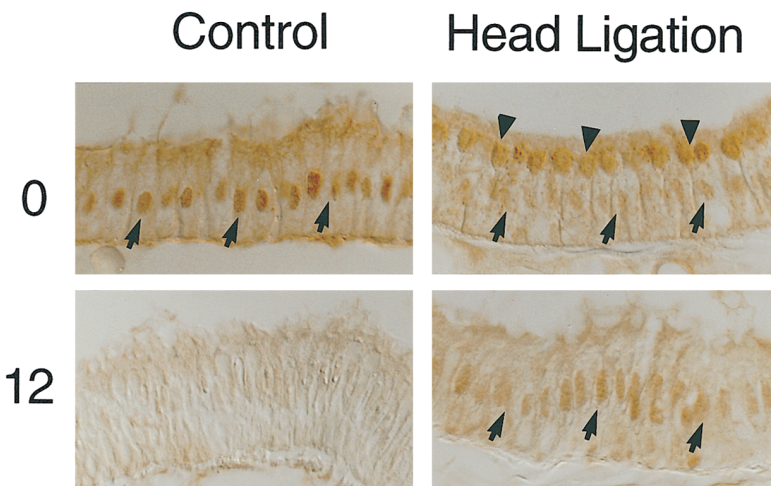

Figure 5. PER Expression in Midgut Epithelium of Intact or Brain-Isolated Larvae

PER immunoreactivity in nuclei (arrows) of midgut epithelium (longitudinal sections) from intact larvae (left column) or brain-isolated larvae (right column) examined at clock times 0 or 12 on day 1 after hatching. Arrowheads denote apical PER staining after head ligation. Magnification, 300×.

nuclei, PER staining was clearly apparent in the apical portion of the epithelial cell cytoplasm, a situation never found in intact or ligation-control animals (Figure 5). The apical PER staining does not represent staining in migrated nuclei, because these apical areas did not stain with Hoechst 33342, a DNA-specific fluorescent dye (data not shown).

Since the prothoracic glands of silkmoths contain a circadian clock, which regulates gut-purging behavior in fifth instar larvae (Mizoguchi and Ishizaki, 1982), a final ligation experiment was done in which only the brain was separated from the rest of the body. Once again, there was an immediate dysregulation of PER movement in the nuclei of midgut epithelium (Figure 4D). This result confirmed that the brain is necessary for the circadian movement of PER into midgut epithelial nuclei.

We next examined whether a transplanted brain would reestablish normal circadian control of PER movement into midgut epithelial nuclei. This seemed a reasonable possibility, given the ability of a transplanted brain to control the circadian gate of egg-hatching behavior. For this experiment, larvae with a head ligation received at the time of ligation a brain transplant of the same circadian phase as the recipient. The results from two experiments showed clearly that the transplanted brain did not normalize the nuclear midgut oscillation (Figure 6). Thus, the humoral influence of the brain is inadequate for circadian control of PER movement into the nuclei of midgut epithelium. Instead, neural connections between brain and midgut appear necessary for this circadian control.

Discussion

The results demonstrate that the circadian gate of egg-hatching behavior is controlled by a brain-centered clock. This result, along with results from our previous *per* antisense experiments in which antisense treatment markedly decreased PER levels in brain and disrupted circadian control of egg-hatching behavior (Sauman et al., 1996), implicates the eight PER-positive cells in embryonic brain as the site of a circadian clock controlling egg-hatching behavior. Thus, the same PER-containing cells in brain may function collectively as a circadian clock throughout the life history of silkmoths (from embryo to adult).

The lack of a detectable rhythm of PER levels in embryonic brain, however, is puzzling. In *Drosophila*, circadian oscillations of PER are thought to be important for the molecule's function as a central clock element (see Reppert and Sauman, 1995; Dunlap, 1996; Hall, 1996). Recent studies with *per* antisense treatments in pharate

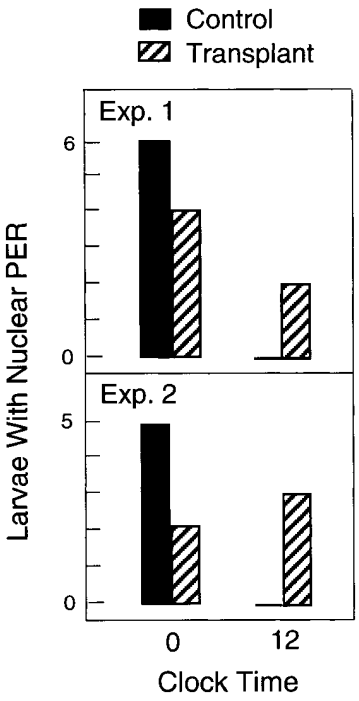

Figure 6. Brain Transplant Does Not Restore Circadian Control of Nuclear PER in Midgut Epithelium

Brains of the same circadian phase were transplanted into the abdomen at the time of ligation. The number of larvae with nuclear PER in midgut epithelium (detected by immunocytochemistry) is depicted for each of two experiments for control (closed vertical bars) and transplanted (hatched vertical bars) larvae examined at clock times 0 and 12. For Experiment 1, six animals were examined for each group at each time. For Experiment 2, five animals were examined for each group at each time.

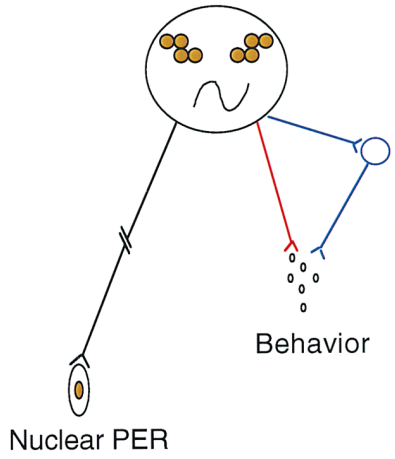

Figure 7. Model of Output Pathways from a Circadian Clock in Embryonic Brain

The brown circles within the larger circle (a collective circadian clock) represent PER-positive cells in the brain of the silkmoth embryo. To the lower right are depicted alternative output pathways leading to release of a brain factor that triggers egg-hatching behavior. The blue lines represent connections between the central clock and potential neurosecretory cells reponsible for releasing the brain factor involved in hatching. The red line represents release of the brain factor directly from the PER-positive neurons. The left depicts a neural pathway (black lines) from brain to midgut responsible for controlling the circadian movement of nuclear PER.

adults suggest that the circadian oscillation of PER in silkmoth brain is important for the circadian regulation of adult eclosion behavior (unpublished data). So why is there a lack of PER oscillations in the brain of the silkmoth embryo? It is still possible that a biologically relevant circadian oscillation of PER exists in embryonic brain that is below the limits of sensitivity of our immunocytochemical methods. Alternatively, oscillating protein levels may not be an essential requirement for PER's function as a central clock element in the embryo. It is also conceivable that PER is not a necessary clock element in embryonic brain, but instead is a critical component of the output pathway that regulates egg-hatching behavior.

An exciting discovery from the transplantation experiments is evidence that a humoral brain factor mediates circadian control of egg-hatching behavior. This could be a factor secreted from the PER-containing cells in brain or from other neurosecretory neurons controlled by the PER-containing cells (Figure 7). Although PER is found in axonal projections in brain cells of first instar larvae after hatching, the peptide lacks a signal sequence and is not detectable in axons prior to hatching. It thus seems unlikely that PER is the humoral factor initiating egg-hatching behavior.

Eclosion hormone (EH) is a neuropeptide that readily comes to mind as a potential egg-hatching trigger. This hormone is important for all ecdysis events in insects (Truman et al., 1981; Truman, 1992). In addition, its release is under circadian control, and EH is expressed in the silkmoth embryo (Truman et al., 1981; unpublished data). However, Truman and colleagues (1981) have shown that although there is a dramatic drop in extractable EH from embryos flanking embryonic ecdysis (which occurs at ~65% of embryogenesis), silkmoth embryos show no sign of depletion of extractable EH at hatching. EH thus seem unlikely to be the egg-hatching trigger. The most provocative possibility is that the humoral factor from brain that initiates egg hatching (tentatively termed "hatchin") is a new, yet-to-be characterized hormone.

Our finding that a brain factor mediates circadian control of egg-hatching behavior in silkmoths is reminiscent of the discovery by Silver and coworkers (1996) that a diffusible substance from transplants of the suprachiasmatic circadian clock can mediate circadian control of locomotor activity in hamsters. Since molecular components appear to be conserved between the circadian clocks of insects and mammals (e.g., *per*), elucidation of a "chronoactive" diffusible substance in an experimentally tractable insect may aid identification of a similar substance in mammals.

Brain control of the nuclear oscillation of PER in midgut epithelium was unexpected and is vastly different from the control of nuclear PER in peripheral tissues in *Drosophila*. In virtually all areas of *Drosophila* in which PER cycles in and out of the nucleus, it has been shown that this cycling is tissue (and probably cell) autonomous (Plautz et al., 1997). Furthermore, PER–TIM dimerization is important for nuclear translocation of PER in fruit flies (see Reppert and Sauman, 1995; Dunlap, 1996; Hall, 1996). In silkmoth larva, on the other hand, we have been unable to detect TIM immunoreactivity in the same midgut epithelial cells in which PER is cycling in and out of the nucleus (Sauman et al., 1996). TIM immunoreactivity is also not detectable in photoreceptor nuclei of the adult eye, the only other tissue in the silkmoth in which nuclear PER has been identified (unpublished data). TIM immunoreactivity is clearly present in the cytoplasm of PER-positive cells in larval and adult brains using the same antibody (Sauman and Reppert, 1996b; Sauman et al., 1996). Thus, the circadian control of nuclear PER in midgut epithelium is extracellular, rather than intracellular, and may not require dimerization with TIM. These findings strongly suggest that the nuclear regulation of PER differs between silkmoths and fruit flies.

Our previous studies have shown that the dynamics of PER regulation in adult silkmoth brain are different from the dynamics of PER regulation in *Drosophila* (Sauman and Reppert, 1996b); in adult silkmoth brain, PER and *per* mRNA levels cycle, but there is no detectable movement of PER into the nucleus. A striking difference in *per* regulation from that described in *Drosophila* has also been recently shown for *per* homologs in mammals. In mammals, *per* RNA levels are regulated acutely by light (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997), while in fruit flies light acutely decreases TIM levels (Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996) with a resultant, secondary phase alteration in the *per* RNA rhythm by the next circadian cycle (Lee et al., 1996). Therefore, based on studies of *per* regulation in *Drosophila*, silkmoths, and mammals, it appears that the same clock element has evolved to subserve circadian function using widely different molecular mechanisms.

Even though the circadian regulation of egg-hatching behavior involves a humoral factor, circadian control of the nuclear movement of PER in midgut epithelium requires an intact (i.e., connected) brain. This shows that in the silkmoth embryo there are at least two different output pathways regulating circadian activities (Figure 7). The requirement of an intact brain for circadian regulation of PER in midgut epithelium further suggests that there is a neural pathway from brain to midgut epithelium involved in its circadian control. Moreover, this neural control is apparent by day 7 of embryogenesis when the circadian oscillation in nuclear PER is first evident in the midgut (Sauman et al., 1996). Midgut epithelium thus provides an interesting model in which to examine the mechanisms regulating an output pathway from a brain-centered clock.

The results presented here provide important insights into clock gene regulation and circadian function during insect embryogenesis. Our findings also add substantially to the rapidly expanding recognition of differences in *per* regulation. It is clear that the more we learn about the molecular mechanisms of circadian clock function in different organisms, the greater will be our understanding of the ways in which circadian clock mechanisms have evolved.

Experimental Procedures

Animals

Diapausing pupae of *A. pernyi* were obtained from Worldwide Butterflies Limited (Sherborne, England) under U. S. Department of Agriculture permit no. 32495. Cocoons were stored in darkness at 4°C until use. To terminate diapause and initiate adult development, the pupae were removed from their cocoons and placed in environmental compartments at 24°C in 17L:7D (Williams, 1969). Adult development was completed within 3–4 weeks, followed by adult eclosion.

Egg Collection and Scoring of Egg-Hatching Behavior

Male and female moths were allowed to mate following adult eclosion. Mated females (paired for ~24 hr) were placed into paper bags and returned to 17L:7D. Following the 7 hr of darkness, the eggs were collected from the inside of the bags and placed individually in 96-well microtiter plates. Typically, one female laid 150–200 eggs on the first night, and only eggs laid on the first night were used. Eggs were maintained at 24°C and 55% relative humidity, in 12L:12D. The dark portion of the lighting cycle consisted of dim red light (wavelength >620 nm, 0.8 $\mu W/cm^2$) from special fluorescent tubes (Litho Light no. 2, Chemical Products), which also remained on during the light phase. Egg hatching was examined at 1 or 2 hr intervals to record the number of larvae hatched during the previous interval. When eggs were examined during the dark portion of the lighting cycle, the same dim red light used for constant darkness aided visual inspection so that the eggs were not exposed to an additional light source.

Transplantation Experiments

Brain or midgut transplantations were performed on pharate larvae 1 day before hatching. The developmental stage of the pharate larvae was determined according to the pigmentation of the cuticle. Brain with retrocerebral complex or midgut was dissected under sterile Ringer's solution from pharate larvae entrained to one of two lighting conditions and implanted through a small opening in the chorion (~200 μm in diameter) into the thoracic or abdominal cavity of the recipient pharate larva. The opening in the chorion of the recipient egg was sealed with a small drop of dental wax. The eggs with the operated pharate larvae were put, one per well, in 96-well microtiter plates and placed in constant darkness. Each experiment was performed at least twice using a set of larvae from different females. Egg hatching was monitored at 1 hr intervals as described above. The mortality rate was ~40% for transplanted pharate larvae and 3% for unoperated animals.

Ligation Experiments

Newly hatched first instar larvae were ligated with surgical silk thread No. 5-0 (Champion USP) either between the head and the first thoracic segment (brain-only isolation) or between the first and second thoracic segment (brain and prothoracic gland isolation). Immediately after ligation, the ligated part of the larval body (head or head with first thoracic segment) was surgically removed to eliminate possible influence of brain and/or prothoracic gland. Ligation between the sixth and seventh larval abdominal segment served as a control. Ligated larvae were returned to LD 12:12 and dissected at 4 hr intervals for immunocytochemistry.

Immunocytochemistry

Larvae were CO_2 anesthetized and immediately fixed in modified Bouin-Hollande solution overnight at 4°C (Sauman and Reppert, 1996a). Standard histochemical techniques were employed for tissue dehydration, embedding in paraplast, sectioning (4–7 μm), deparaffinization, and rehydration. The sections were then treated with Lugol's iodine followed by 7.5% solution of sodium thiosulfate to remove residual heavy metal ions from the fixed tissue. After thorough washing with distilled water and PBS supplemented with 0.2% Tween 20 and 0.1% bovine serum albumin (PBS-TB), the sections were blocked with 10% normal goat serum in PBS-TB (30 min at room temperature) and incubated with desired primary antibody (appropriately diluted in PBS-TB) in a humidified chamber overnight at 4°C. Following thorough rinsing with PBS-TB (three times for 10 min at room temperature), sections were incubated with goat anti-rabbit IgG–horseradish peroxidase (HRP) conjugated secondary antibody (Jackson ImmunoResearch, 1:1000 in PBS-TB, 1 hr at room temperature). The HRP enzymatic activity was visualized with hydrogen peroxide (0.005%) and 3,3′-diaminobenzidine tetrahydrochloride (0.25 mM in 0.05 M Tris-HCl [pH 7.5]) as a chromogen. Stained sections were dehydrated and mounted in AccuMount-60 mounting medium. In all cases, the staining obtained by immunocytochemistry with diaminobenzidine was verified using immunofluorescence (goat anti-rabbit Cy3 conjugated secondary antibody, 1:800 in PBS-TB, Jackson ImmunoResearch). The mounted specimens were viewed and photographed under a Olympus BX60 microscope equipped with Nomarski (DIC) optics and epifluorescence.

The following primary antibodies and their appropriate dilutions in PBS-TB were used for immunocytochemistry: rabbit anti-*A. pernyi* PER (57/10w at 1:500; Sauman and Reppert, 1996b); rabbit anti-*A. pernyi* PER (58/10w at 1:500; Sauman and Reppert, 1996b); and rabbit anti-αPER (*D. melanogaster* Baculovirus recombinant protein from M. Young, Rockefeller University, dilution 1:1000).

In control experiments, the primary antibodies were replaced with normal goat serum. As an additional control for binding specificity, the anti-*A. pernyi* primary antibodies (57/10w and 58/10w) were preincubated with 100 molar excess of their original antigens prior to immunological staining. In all cases, no significant staining above background was observed.

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