Oviposition-promoting pars intercerebralis neurons show *period*-dependent photoperiodic changes in their firing activity in the bean bug

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Animals show photoperiodic responses in physiology and behavior to adapt to seasonal changes. Recent genetic analyses have demonstrated the significance of circadian clock genes in these responses. However, the importance of clock genes in photoperiodic responses at the cellular level and the physiological roles of the cellular responses are poorly understood. The bean bug *Riptortus pedestris* shows a clear photoperiodic response in its reproduction. In the bug, the pars intercerebralis (PI) is an important brain region for promoting oviposition. Here, we analyzed the role of the photoperiodic neuronal response and its relationship with clock genes, focusing on PI neurons. Large PI neurons exhibited photoperiodic firing changes, and high firing activities were primarily found under photoperiodic conditions suitable for oviposition. RNA interference-mediated knockdown of the clock gene *period* abolished the photoperiodic response in PI neurons, as well as the response in ovarian development. To clarify whether the photoperiodic response in the PI was dependent on ovarian development, we performed an ovariectomy experiment. Ovariectomy did not have significant effects on the firing activity of PI neurons. Finally, we identified the output molecules of the PI neurons and analyzed the relevance of the output signals in oviposition. PI neurons express multiple neuropeptides—insulin-like peptides and diuretic hormone 44—and RNA interference of these neuropeptides reduced oviposition. Our results suggest that oviposition-promoting peptidergic neurons in the PI exhibit a circadian clock-dependent photoperiodic firing response, which contributes to the photoperiodic promotion of oviposition.

photoperiodism | circadian clock | pars intercerebralis | insulin-like peptides | diuretic hormone 44

Animals in a seasonally fluctuating environment modulate their physiological status and behavior according to the photoperiod for seasonal adaptation. Since Bünning (1) proposed the idea of photoperiodic time measurement based on circadian rhythms, research on the significance of circadian clock systems in photoperiodic responses has advanced. An increasing number of studies using different species have demonstrated the involvement of circadian clock genes in the photoperiodic response to propose that the circadian clock system contributes to time measurement for photoperiodic responses in both vertebrates (birds and mammals) and invertebrates (insects) (2–5). Advanced genetic studies in insects have reported that the suppression of clock-gene expression diminishes photoperiodic responses such as short-day-induced diapause and temperature tolerance (6–11). Therefore, it is becoming increasingly accepted that the molecular circadian clock underlies physiological and behavioral photoperiodic responses. To understand the photoperiodic mechanism, it is necessary to know how the clock genes or systems affect the brain neurons that control physiology or behavior. However, there are few studies investigating photoperiodic responses at the cellular level in brain networks (12–14), and the importance of clock genes in photoperiodic cellular responses has not been analyzed. Thus, to understand the photoperiodic mechanisms controlling physiology and behavior, understanding the cellular response to photoperiod in brain neurons linking the circadian clock systems to the physiological output is crucial.

Here, we analyzed the cellular photoperiodic response involving clock-gene expression in the brain of the bean bug *Riptortus pedestris*. Five circadian clock genes have been identified in *R. pedestris* (15, 16). This insect shows clear photoperiodic responses in reproduction, and RNA interference (RNAi)-mediated knockdown of the clock genes has been shown to attenuate reproductive photoperiodism (10, 11, 16). Additionally, neuroanatomical analyses have shown that some neurosecretory cells projecting to the endocrine organs, the corpus cardiacum–corpus allatum, play an important role in controlling reproduction (17, 18). Because of its genetic and neuroanatomical background, this insect is a good model for investigating the cellular photoperiodic responses based on clock genes in the neurosecretory cells that control reproduction.

In this study, we focused on neurosecretory cells in the pars intercerebralis (PI). The PI is a brain region in which various types of neurosecretory cells involved in endocrine control are localized (19). Because of developmental and functional similarities, the PI is thought to be homologous to the vertebrate hypothalamus, which acts as a neuroendocrine center (20). In *R. pedestris*, previous studies reported that ablation of the PI reduces the number of eggs laid, but does not have a significant effect on the photoperiodic response in ovarian development, and transplantation of the PI rescues this reduction (17, 18). In some other insects, ablation of PI cells also results in reduced fecundity (21, 22). Thus, the PI neurosecretory cells may respond to photoperiod to change fecundity.

Here, we investigated the clock-gene-dependent photoperiodic response in PI cells by electrophysiological and genetic techniques.

Significance

Animals adapt to seasonal changes by photoperiodic modulation of their physiology and behavior. It is widely recognized that circadian clock systems underlie photoperiodic regulation. However, the importance of clock genes in photoperiodic responses at the cellular level is poorly understood. In this study, we focused on the insect neuroendocrine center, pars intercerebralis (PI), which is known to be homologous to the vertebrate hypothalamus. A combination of electrophysiological and genetic analyses revealed that oviposition-promoting PI neurons showed photoperiodic responses dependent on the clock gene *period*. This report shows the significance of a clock gene in cellular photoperiodic responses. The present analyses are a good model for revealing clock-gene-dependent photoperiodic responses at the cellular level.

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We found that the large PI neurons exhibited a clear photoperiodic response in their spontaneous firing activity. The photoperiodic firing response was abolished by RNAi-mediated knockdown of the clock gene *period* (*per*). We also demonstrated that RNAi of neuropeptides, insulin-like peptides (ILPs) and diuretic hormone 44 (DH44), which are expressed in large PI cells, reduces oviposition. These findings suggest the significance of the circadian clock-gene expression in the photoperiodic firing response and the physiological role of the PI response in oviposition.

Results

PI Neurons Change Their Spontaneous Firing Activity According to the Day Length and Days after Eclosion. To reveal the photoperiodic response at the cellular level, we compared the firing activity of PI neurons between day lengths at different days. We first examined ovarian development and oviposition in females. On day 5 (5 d after eclosion), most females had immature ovaries (diapause), and oviposition was not found under both long-day (16-h light:8-h dark) and short-day (12-h light:12-h dark) conditions (*SI Appendix*, Fig. S1). In contrast, on day 20–22 (20 to 22 d after eclosion), many females had mature ovaries (nondiapause), and more than half oviposited under the long-day condition, whereas most females were immature and did not oviposit under the short-day condition (*SI Appendix*, Fig. S1).

In these four groups, we recorded the spontaneous firing activity of PI neurons in the daytime (zeitgeber time [ZT]0–12). In all four groups, PI neurons showed a variety of spontaneous firing activities, and we classified the activities into three types: burst (high-frequency firing), nonburst (low-frequency firing), and silent (Fig. 1*A*). In long-term recorded samples, some neurons exhibited the continuous burst firings (for several tens of seconds to several minutes) with low-activity intervals, and some neurons showed a shift in firing patterns (*SI Appendix*, Fig. S2). In the present study, we uniformly analyzed the spontaneous

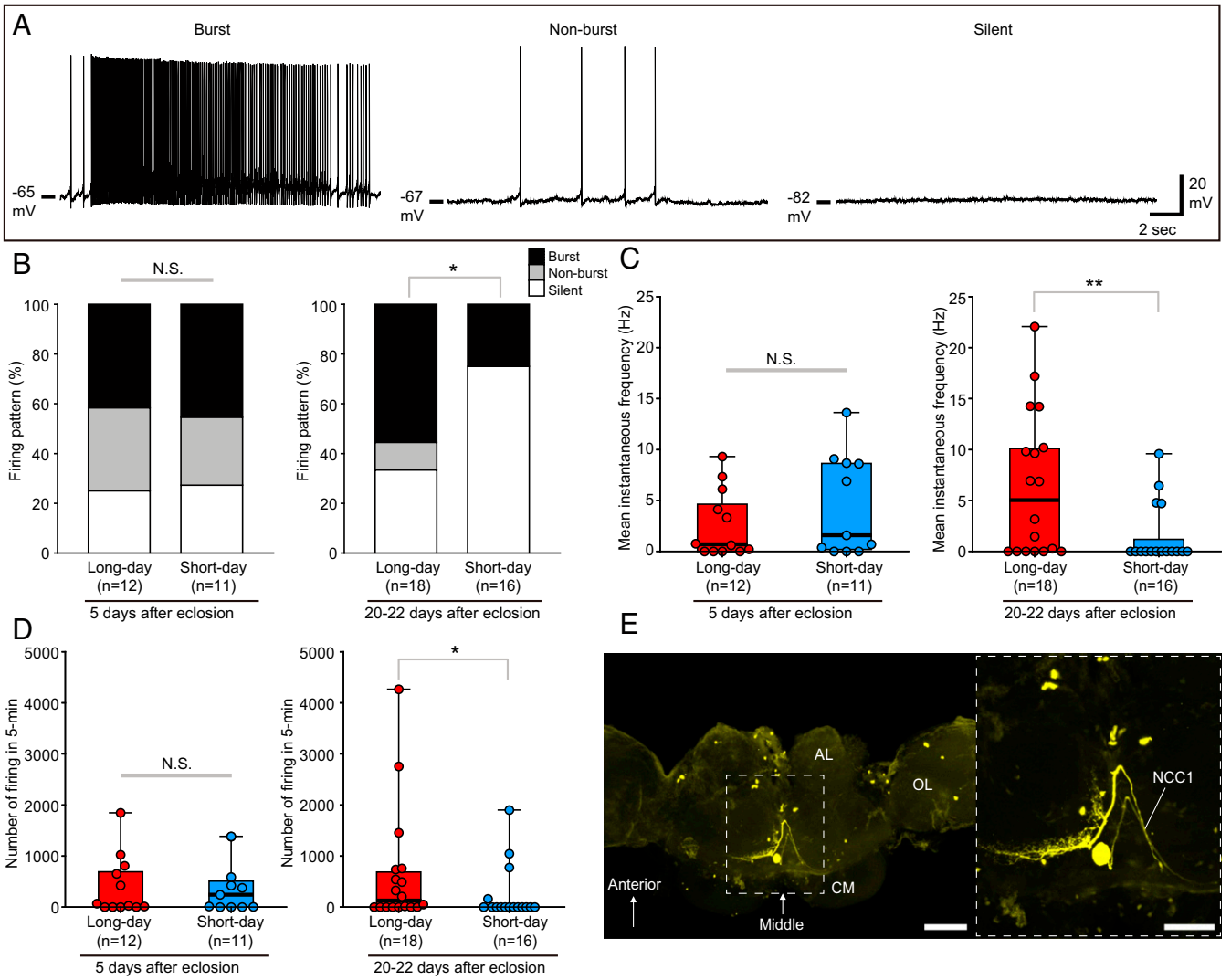

Fig. 1. PI neurons change their spontaneous firing activity according to the day length and number of days after eclosion. (*A*) Representative traces showing burst, nonburst, and silent activities in PI neurons. (*B*) Proportions of the three firing patterns among the four groups with different day-length/number-of-days conditions. (*C* and *D*) Box plots with scatterplots showing the mean instantaneous frequency (*C*) and number of firing in 5 min (*D*) among the four groups with different day-length/number-of-days conditions. (*E*) Images in dorsal view showing the representative projections from the recorded PI neuron. The PI neuron extends dendrites ipsilaterally near the cell body and projects the axon to the contralateral NCC1. (*B*) χ^2 test. (*C* and *D*) Mann–Whitney *U* test. $*P < 0.05$; $**P < 0.01$. N.S., not significant. (*C* and *D*) The lines at the top, middle, and bottom of box plots show the upper quartile, median, and lower quartile, respectively. The upper and lower whiskers of the box plots show the maximum and minimum values, respectively. (*E*) AL, antennal lobe; CM, calyx of mushroom body; OL, optic lobe. (Scale bars: 100 μm [*Left*] and 50 μm [*Right*].)

activity 5 to 10 min after the start of recording, which was a stable period for reliable recording. On day 5, there was no significant difference in the firing-pattern proportion between the long-day and short-day conditions (Fig. 1*B*). The instantaneous frequency and number of firing did not differ significantly between the long-day and short-day conditions on day 5 (Fig. 1 *C* and *D*). In contrast, on day 20–22, more than half of the PI neurons showed high-frequency burst activity under the long-day condition, whereas 75% of them showed a silent pattern under the short-day condition (Fig. 1*B*). There was a significant difference in the firing-pattern proportion between the long-day and short-day conditions on day 20–22 (Fig. 1*B*). Even in the short-day condition on day 20–22, a few PI neurons showed the burst firing, and some females developed ovaries (Fig. 1*B* and *SI Appendix*, Fig. S1). Because burst firing occurred at a similar rate in females with developed and undeveloped ovaries under the short-day condition (*SI Appendix*, Table S1), occurrence of the burst firing seems not related to the ovarian development. On day 20–22, the instantaneous frequency and number of firing in the long-day condition were higher than those in the short-day condition (Fig. 1 *C* and *D*). These results clearly show that PI neurons changed their spontaneous firing activity according to the day length on day 20–22, but not on day 5. We also examined the PI firing activity on day 20–22 in the nighttime (ZT16–24). In the nighttime also, many PI neurons under the long-day condition showed the high spontaneous firing activity, whereas most PI neurons under the short day were silent (*SI Appendix*, Fig. S3). Photoperiodic change in the firing activity was observed both in the daytime and nighttime.

Recorded neurons extended dendrites ipsilaterally near the cell body and projected axons to the contralateral nervi corporis cardiaci 1 (NCC1) (Fig. 1*E*), which was the same as the projections of PI neurons previously reported (17). This result supports that the recorded cells were PI cells.

RNAi-Mediated Knockdown of *per* Diminishes the Photoperiodic Response in PI Neurons. A previous study reported that RNAi of the circadian clock gene *per* averts the reproductive diapause under the short-day condition (11). Thus, we next analyzed the significance of *per* in the photoperiodic response of PI neurons in the long-term photoperiod treatment (on day 20–21) by double-stranded RNA (dsRNA)-induced RNAi. We prepared dsRNA for *β-lactamase* (ds*bla*)-injected females for control and *per* (ds*per*)-injected females in the long- and short-day conditions. Similar to intact females on day 20–22 (*SI Appendix*, Fig. S1), most control ds*bla*-injected females were nondiapausing and oviposited under the long-day condition, but not under the short-day condition (Fig. 2 *A*–*C*). Contrastingly, ds*per*-injected females developed their ovaries and oviposited even under the short-day condition, and there were no significant differences in ovarian development and oviposition between the long-day and short-day conditions (Fig. 2 *A*–*C*). *per* expression in the brain of the ds*per* females was significantly lower than that of ds*bla* females, although the decreased value was small (~10 to 20%) (*SI Appendix*, Fig. S4). These results demonstrated that ds*per* injection clearly abolished the photoperiodic response in ovarian development and oviposition.

In these dsRNA-injected females, we recorded the spontaneous firing activity of PI neurons. Similar to the results for intact cells, more than 60% of PI neurons in the control ds*bla*-injected females showed burst activity under the long-day condition, whereas the activity of most PI neurons was silent under the short-day condition (Fig. 2*D*). In the ds*bla*-injected females, the instantaneous frequency and number of firing under the long-day condition were higher than those under the short-day condition (Fig. 2 *E* and *F*). These results demonstrated that PI neurons in the control ds*bla* group showed a photoperiodic response similar to that of intact females. In contrast, many PI neurons of ds*per*-injected females showed high-frequency burst activity in the short-day condition, in addition to the long-day condition (Fig. 2*D*). In the ds*per* group, there were no significant differences in the firing-pattern proportion, instantaneous frequency, and number of firing between the long-day and short-day conditions, and the values were not different from those in ds*bla*-injected females under the long-day condition (Fig. 2 *D*–*F*). These results show that the RNAi of *per* diminished the photoperiodic response in the PI firing activity.

Ovariectomy Has No Significant Effects on PI Firing Activity under the Long Day. The RNAi of *per* attenuated the photoperiodic response both in the firing activity of PI cells and ovarian development (Fig. 2). Thus, it was possible that the effect of *per* knockdown on PI neurons was indirectly caused by changes in ovarian development. To examine whether ovarian development affects PI firing activity, we analyzed the effects of ovariectomy (OVX; Fig. 3*A*) on PI firing activity under long-day conditions on day 20–22. We analyzed the effects of surgery performed on two different days: 1 d after eclosion (day 1 operation: ovary was immature) or 10 d after eclosion (day 10 operation: ovary was mature). In both day 1 and day 10 operations, approximately half the PI neurons in the sham-operated females, whose ovaries were mature, exhibited burst activity similar to the intact females (Fig. 3*B*). In the OVX females, 50% or more of PI neurons exhibited burst firing, and there was no significant difference in the firing proportion between the sham- and OVX-operated females within both day 1 and day 10 operations (Fig. 3*B*). We also compared the instantaneous frequency and number of firing and found that they were not significantly different between the sham- and OVX-operated females (Fig. 3 *C* and *D*). These results indicate that high PI firing activity under long-day conditions was not caused by ovarian maturation.

Gene-Expression Profiling of Clock Genes and Output Molecules in Large PI Neurons. We next asked whether the PI neuron itself expresses *per* and other clock genes. It is known that the large PI cells express *per* in some insects (23–26). In addition to *per*, *cycle* (*cyc*), *vrille* (*vri*), and *mammalian-type cryptochrome* (*cry-m*) have been identified as clock genes and suggested to work together for the circadian clock in *R. pedestris* (15). However, clock-gene-expressing cells are not identified in *R. pedestris*. Then, we analyzed whether clock genes are expressed in the large PI neurons of *R. pedestris*. We collected 8 PI cells each from 5 females (total 40 cells) and performed single-cell RT-PCR (Fig. 4*A*). All collected PI cells expressed the housekeeping gene *beta-tubulin* (*tubulin*) as a positive control (Fig. 4*B* and *SI Appendix*, Table S2). In each individual, 62.5 to 87.5% of PI cells expressed at least one of the clock genes, and 40% of the large PI cells expressed *per*. However, PI cells did not express a full set of clock genes, except for one cell (Fig. 4*B* and *SI Appendix*, Table S2). These suggest that most large PI cells are not working as clock cells, whereas it remains a possibility that a small subset of PI cells act as clock cells.

Considering that PI neurons are known to release different kinds of neuropeptides in some dipterans (27, 28), we hypothesized that PI neurons in *R. pedestris* photoperiodically change the firing activity to regulate the release of neuropeptides. Thus, we also analyzed expression of neuropeptides, DH44 and ILPs (*SI Appendix*, Fig. S5), which are known to be expressed in *Drosophila melanogaster* PI neurosecretory cells (28). Almost all the large PI cells expressed *Ilp1*, and 80%, 50%, and 17.5% of the large PI cells expressed *Dh44*, *Ilp2a*, and *Ilp2b*, respectively (Fig. 4*B* and *SI Appendix*, Table S2). These results suggest that the large PI neurons mostly expressed ILPs and DH44 as output molecules.

ILP1 and 2, and DH44 Expressed in the Large PI Neurons Are Involved in Promoting Oviposition. It has been reported that the PI is involved in promoting oviposition under long-day conditions (18). The high firing activity under long-day conditions on day 20–22

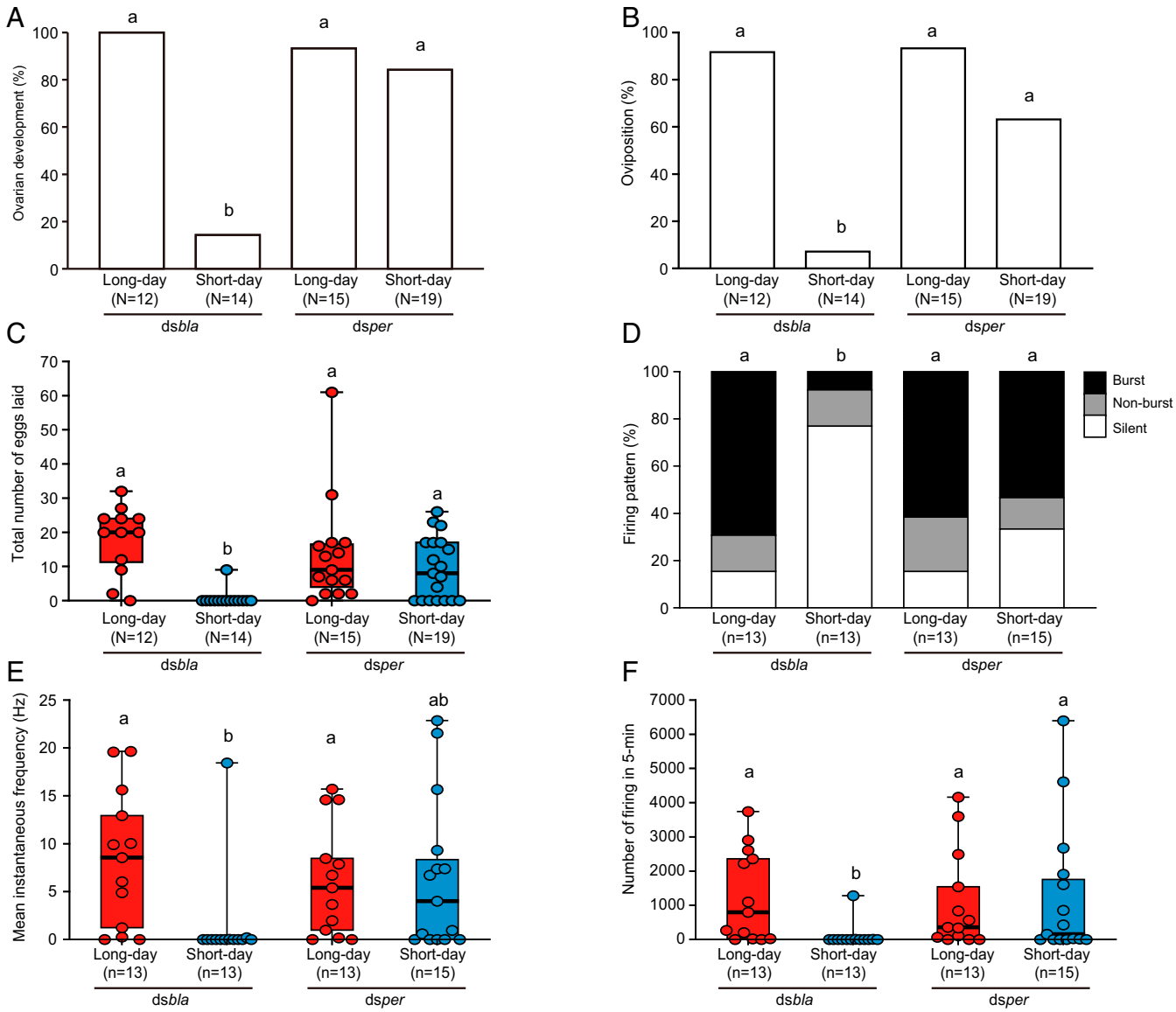

Fig. 2. RNAi of *per* diminishes the photoperiodic responses in the ovarian development and PI firing activity. Data were obtained in ds*bla*- and ds*per*-injected females under the long-day and short-day conditions on day 20–21. (*A*) Proportions of ovarian development. (*B*) Proportions of oviposition. (*C*) Box plots with scatterplots showing total number of eggs laid. (*D*) Proportions of the three firing patterns in large PI cells. (*E* and *F*) Box plots with scatterplots showing the mean instantaneous frequency (*E*) and number of firing in 5 min of the PI cells (*F*). Columns and box plots with different letters indicate statistically significant differences. (*A*, *B*, and *D*) Tukey-type multiple comparisons for proportions (*D*, silent-rate comparison), (*C*, *E*, and *F*) Steel–Dwass test: $P < 0.05$. The lines at the top, middle, and bottom of box plots show the upper quartile, median, and lower quartile, respectively. The upper and lower whiskers of the box plots show the maximum and minimum values, respectively. (*A*–*C*) N, number of females. (*D*–*F*) n, number of recorded PI cells.

(Fig. 1) suggests that the large PI neurons release neuropeptides (ILPs and DH44) in accordance with day length to control oviposition. To test this, we examined whether these neuropeptides are involved in oviposition by RNAi-mediated knockdown. Injections of dsRNA for *Dh44* (ds*Dh44*), *Ilp1* (ds*Ilp1*), and *Ilp2* (ds*Ilp2*) specifically reduced the expression level of each target gene (*SI Appendix*, Fig. S6). The number of eggs laid in the ds*Dh44*-, ds*Ilp1*-, and ds*Ilp2*-injected females was significantly fewer than that in the control ds*bla*-injected females (Fig. 5 *A* and *B*). These results suggest that DH44, ILP1, and ILP2 play an important role in promoting oviposition. We also examined ovarian development, gonadosomatic index, body-weight change, and hemolymph reducing sugar levels. RNAi of *Dh44*, *Ilp1*, and *Ilp2* had no significant effect on ovarian development, gonadosomatic index, and body-weight change (Fig. 5*C* and *SI Appendix*, Fig. S7). However, the hemolymph reducing sugar level in the ds*Ilp1*-injected females was significantly higher than that in ds*bla*-injected females, and ds*Ilp2*- and ds*Dh44*-injected females also showed a trend of increased hemolymph reducing sugar level (Fig. 5*D*).

Discussion

In mammals, the suprachiasmatic nucleus (SCN), known as the master clock for circadian rhythms, encodes photoperiodic information via changes in the neural plasticity of the SCN network (29–34). In the cockroach *Leucophaea maderae*, circadian pacemaker neurons in the optic lobe change their cell number and branching patterns according to photoperiod (35). Some electrophysiological studies in insects have shown photoperiodic changes in neurosecretory cells at the neuronal activity level (12,

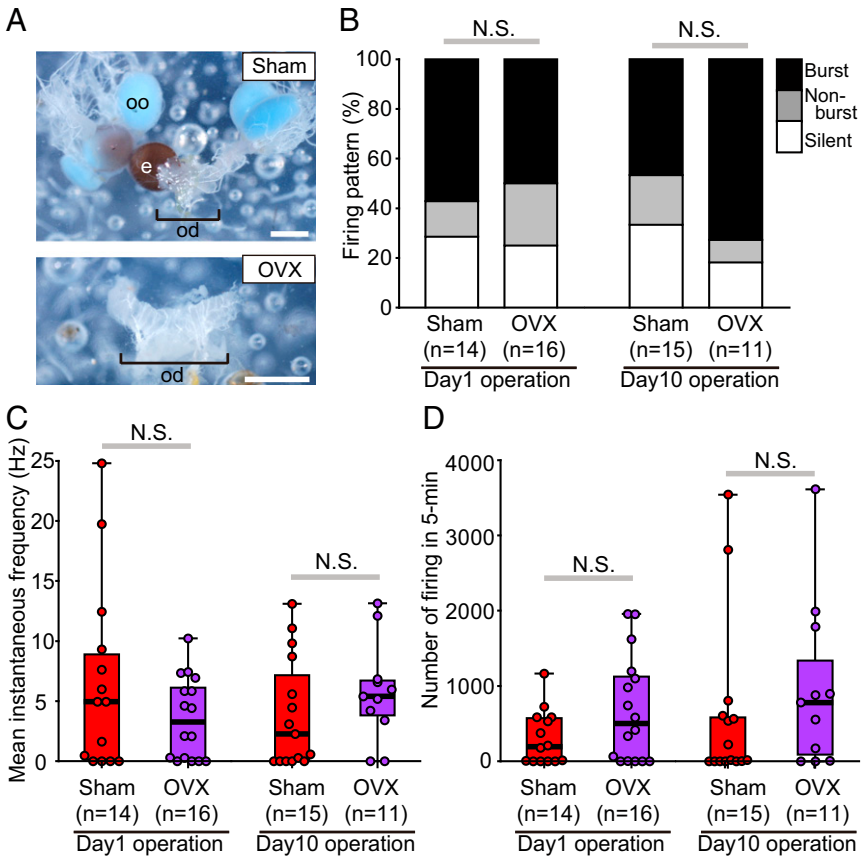

Fig. 3. Ovariectomy (OVX) has no significant effects on the firing activity of PI neurons under the long-day conditions in both day 1 and day 10 operations. (*A*) Representative images of the ovary in the sham (*Upper*) and OVX (*Lower*) females. (*B*) Proportions of the three firing patterns in the sham and OVX females. (*C* and *D*) Box plots with scatterplots showing the mean instantaneous frequency (*C*) and number of firing in 5 min (*D*) in the sham and OVX females. The lines at the top, middle, and bottom of box plots show the upper quartile, median, and lower quartile, respectively. The upper and lower whiskers of the box plots show the maximum and minimum values, respectively. (*B*) χ^2 test. (*C* and *D*) Mann–Whitney *U* test. N.S., not significant. (*A*) e, egg; od, oviduct; oo, oocyte. (Scale bars: 1 mm.)

13). These studies suggested that photoperiodic encoding occurs at either the single-cell or network level. However, it is unclear whether clock genes are involved in photoperiodic encoding at the cellular level. The present study revealed that PI neurons enhance their firing activity under long-day conditions leading to oviposition, and the clock gene *per* is essential for the photoperiodic change in the firing activity of PI neurons. This report shows the importance of the clock genes in the photoperiodic responses at cellular levels.

The large PI neurons in *R. pedestris* exhibited three firing-activity patterns: burst, nonburst, and silent. Different firing-activity patterns in the PI have also been reported in the cockroaches *Diploptera punctata* (36) and *Periplaneta americana* (37); the cricket *Teleogryllus commodus* (38); and *D. melanogaster* (39), suggesting that neurons with various electrophysiological properties are localized in the PI. The present analyses revealed that the firing proportion in the PI is changed by the long-term photoperiodic treatment; the proportion of the burst type remained high under the long-day condition, whereas the short-day condition increased silent cells. Single-cell PCR and RNAi experiments showed that the large PI neurons are peptidergic neurons expressing at least three neuropeptides (ILP1, 2, and DH44) that promote oviposition. High-frequency firing activity in peptidergic neurons has been reported to be required for the release of neuropeptides (40–45). In vertebrate and invertebrate peptidergic neurons, changes in the firing-pattern proportion occur according to various physiological events/conditions to modulate physiology and behavior (46–50). Based on these reports and our present results, we consider that the day-length-dependent shift of the firing pattern in the PI population may contribute to the modulation of neuropeptide release (ILPs and DH44) and subsequent egg laying.

It is interesting to analyze the cellular mechanism underlying change of the firing pattern. The firing pattern may be mainly controlled by intrinsic ionic conductance, or synaptic/peptidergic input from other cells. By comparing the three types of firing activity, the resting membrane potential (RMP) of the high-frequency burst type was more depolarized than that of nonburst and silent types (*SI Appendix*, Fig. S8). Thus, intrinsic ionic channels and transporters regulating the RMP are candidates for the photoperiodic control of the PI firing patterns. In contrast, considering the firing shift in some vertebrate neurons (46, 48), it remains possible that the PI neuron-firing pattern is dependent on synaptic/peptidergic inputs. We plan to identify the photoperiodic factors regulating the spontaneous firing activity of PI neurons in future studies.

Knockdown of the clock gene *per* diminished the photoperiodic response in large PI neurons in *R. pedestris*. In situ hybridization studies have reported that *per* is expressed in large neurosecretory cells in the PI of the linden bug *Pyrrhocoris apterus* (23), the silkworm *Bombyx mori* (24), the Mediterranean flour moth *Ephestia kuehniella* (25), and the migratory monarch butterfly *Danaus plexippus* (26). Single-cell PCR showed that 40% of the large PI neurons in *R. pedestris* expressed *per*. However, we found that most PI cells do not express a full set of clock genes. Previous studies in *R. pedestris* showed that RNAi of *per*, *cyc*, and *cry-m* disrupts the

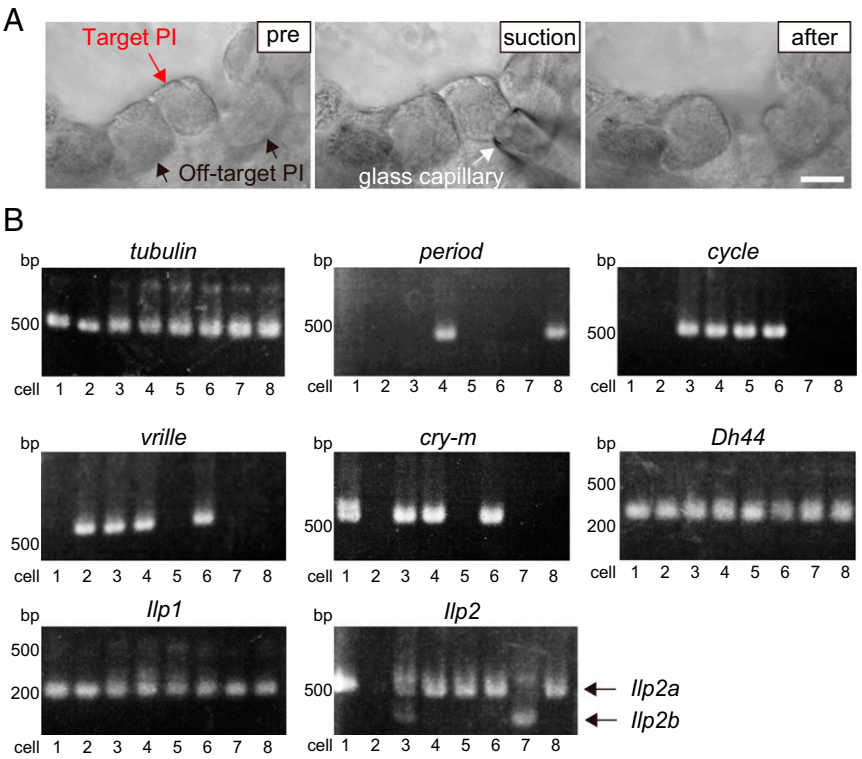

Fig. 4. Detection of mRNA expression in the large PI cells by single-cell reverse-transcription nested PCR. (*A*) A single PI cell was collected by glass capillary. The glass capillary was attached to a target PI cell, and we suctioned the PI cell (*Center*). After suction, we confirmed that only the target cell was collected (*Right*). (Scale bar: 20 μm.) (*B*) Representative images show *tubulin*, *period*, *cycle*, *vrille*, *cry-m*, *Dh44*, *Ilp1*, and *Ilp2* expression in eight PI cells from a single female (female e in *SI Appendix*, Table S2). Because *Ilp2* has an alternative RNA-splicing site, bands were found at two different length positions. We classified the long one as *Ilp2a* (about 530 bp) and the short one as *Ilp2b* (about 350 bp).

cuticle-deposition rhythm (11, 51), although *vri* has not been analyzed. This suggests that, at least, *per*, *cyc*, and *cry-m* may be the core clock components in the *R. pedestris*. Based on the present results and these reports, we think PI cells themselves are not clock cells involved in photoperiodic time measurement, but receive information from other *per*-expressing neurons directly or indirectly to show the photoperiodic response in the firing pattern. However, there remains a possibility that RNAi directly affected some PI cells expressing *per*.

At the anterior proximal medulla of the optic lobe, a region containing pigment-dispersing factor immunoreactive cells is a prerequisite for the photoperiodic response in reproductive diapause (52). In many insects, a circadian master clock is localized in the proximal medulla of the optic lobe (53). Thus, we currently hypothesize that essential clock cells for photoperiodic time measurement are localized in the proximal medulla of the optic lobe. The medulla clock neurons may send photoperiodic information to the PI neurons through multiple synapses. To reveal the molecular basis of how synaptic inputs from other clock cells generate photoperiodic responses in the PI, we would like to perform cell-specific clock-gene manipulation to analyze the significance of input from other clock cells in the PI photoperiodic response.

Although the decrease in the brain *per* expression was small, the present RNAi of *per* clearly attenuated the photoperiodic responses of the PI firing and ovarian development. This suggests a possibility that the injected ds*per* affected only a small number of *per*-expressing cells (such as cells near the surface of the brain), and the *per* expression of the whole brain was not completely reduced. Considering the fact that the photoperiodic response was completely abolished by the present RNAi of *per*, it is possible that injected ds*per* might affect at least the clock cells important for photoperiodism. We also consider another possibility that the slight change in *per* expression plays an important role in the photoperiodic time-measurement. A previous study showed that the *per* expression of the whole head of *R. pedestris* is slightly higher under the short-day condition than under the long-day condition (15). Thus, there is a possibility that this slight up-regulation of *per* under the short-day condition is important for inducing diapause, and the present small knockdown of *per* aborted diapause under the short-day condition.

The PI is involved in multiple endocrine regulatory functions and contains various types of neurosecretory cells (19). In the present study, we found that the large PI neurons of *R. pedestris* express *Ilp1*, *Ilp2*, and *Dh44*. RNAi of *Ilps* and *Dh44* induced a reduction in egg-laying, suggesting that ILPs and DH44 have the physiological effect of promoting egg-laying. Besides suppression of egg-laying, RNAi of *Ilp1* caused hyperglycemia, and that of *Ilp2* also showed a trend. ILPs in *D. melanogaster* (*Drosophila*-ILPs [DILPs]) are known regulators of the hemolymph reducing sugar (glucose) level (54, 55), which is similar to mammalian insulin. In *D. melanogaster*, the cell-specific ablation of DILP cells in the PI has been shown to reduce the number of eggs laid and increased the hemolymph glucose level simultaneously (55). In *Caenorhabditis elegans*, high-glucose treatments significantly suppress the rate and number of eggs laid (56, 57). Although we did not specifically examine *Ilps*-expressing cells in *R. pedestris*, based on the present results and previous studies in other species, we propose that PI neurons producing ILPs contribute to increased egg-laying and control of the sugar circulation in *R. pedestris*. In addition to the ILPs, the PI neurons also express DH44. In female *D. melanogaster*, DH44 neurons in the PI control sperm storage and ejection, and RNAi of *Dh44* also decreases the eggs laid (58). Thus, we consider that DH44-expressing PI neurons

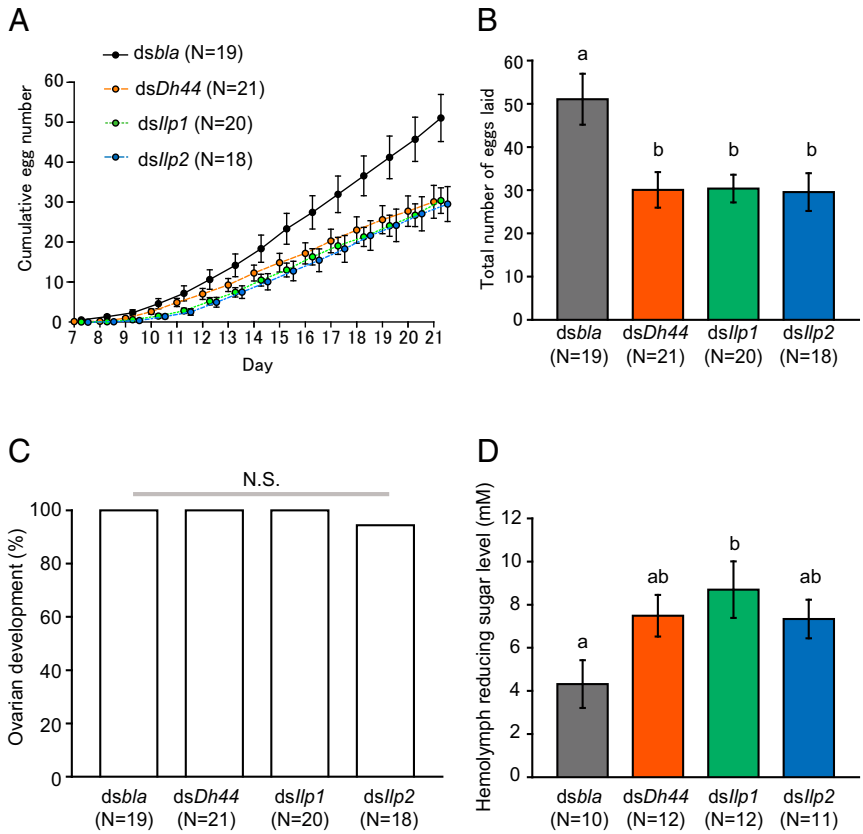

Fig. 5. RNAi of *Dh44*, *Ilp1*, and *Ilp2* reduces egg-laying and increases the hemolymph reducing sugar level, but does not affect ovarian development under the long-day condition. (*A*) The graph showing the mean cumulative number of eggs laid in each dsRNA-injected group between 7 and 21 d after eclosion. (*B*) Mean total number of eggs laid in the ds*bla*-, ds*Dh44*-, ds*Ilp1*-, and ds*Ilp2*-injected groups. (*C* and *D*) Proportion of ovarian development (*C*) and the mean hemolymph reducing sugar level (*D*) in the ds*bla*-, ds*Dh44*-, ds*Ilp1*-, and ds*Ilp2*-injected groups. Columns with different letters indicate statistically significant differences. (*B* and *D*) Tukey–Kramer test. (*C*) Tukey-type multiple comparisons for proportions; $P < 0.05$. N.S., not significant). (*A*, *B*, and *D*) Circles and columns with error bars show mean ± SE.

of female *R. pedestris* may modulate egg-laying through sperm storage and ejection, similar to *D. melanogaster*. Although the target site for ILPs and DH44 in *R. pedestris* has not been identified, we propose that the PI neurons act as promoting egg-laying through the release of ILPs and DH44.

In summary, electrophysiological recordings from brain neurons in gene-knockdown insects demonstrated the significance of the clock gene *per* in the photoperiodic response of single PI neurons. Despite the importance of circadian clock genes in photoperiodic responses, it has been unclear how photoperiodic information is encoded at the cellular or circuit level based on the clock genes. The combination of electrophysiological and genetic methods established in the present study will contribute to clarifying the importance of the molecular basis of the circadian clock in photoperiodic encoding in a single cell.

Materials and Methods

Insects. We used a strain of *R. pedestris* collected in Machikaneyama (36°48′ N, 135°27′E, Toyonaka, Japan) and maintained for 5 to 13 generations. The insects were reared on soybeans, red clover seeds, and water supplemented with L-ascorbic acid sodium salt (0.05%) and L-cysteine (0.025%). Insects were kept under short-day conditions (12-h light/12-h dark) at 25 ± 2 °C before eclosion. After eclosion, the insects were maintained separately for the short-day or long-day conditions (16-h light/8-h dark) at 25 ± 2 °C. We examined the ovarian stage of each female. Ovarian stages were classified from "0" to "V," and we regarded females with ovaries in stages 0, I, and II as reproductive diapause and females with ovaries in stages III, IV, and V as nondiapause (59).

RNAi by dsRNA. To make dsRNAs, we extracted and purified template RNA from the whole head using the FastGene RNA Basic Kit with DNaseI set (Nippon Genetics). Complementary DNA (cDNA) fragments containing the T7 promoter were amplified by using the PrimeScript RT-PCR Kit (Takara Bio Inc.). Based on the amplified cDNA fragments, dsRNAs were synthesized by using the T7 RiboMAX Express RNAi System (Promega). Primer sets for amplifying the cDNA containing the T7 promoter are shown in *SI Appendix*, Table S3. The primers for *bla* and *per* were created by referring to previous studies (11, 60). The concentration of dsRNA was adjusted to 1 μg/μL, with pure water as the working solution. Female insects within 24 h of eclosion were placed on ice, and 1 μL of the working dsRNA solution was injected into their head. After dsRNA injection, females were separately kept in a plastic cup (diameter: 10 cm; depth: 4 cm) under long-day or short-day conditions. To analyze the effects of the RNAi on *per* expression, we extracted total RNA from the whole brain 3 d after eclosion at ZT1–2 (the start time of the light period corresponds to ZT0) and ZT 16–17 under the short day, and purified by the FastGene RNA Basic Kit with DNaseI set (Nippon Genetics). To examine expression of *Dh44*, *Ilp1*, and *Ilp2*, total RNA from the whole head under the long day was extracted 21 d after eclosion at ZT2–5 by the same kit. We measured messenger RNA (mRNA) expression by real-time qPCR. Methods of real-time qPCR are described in *SI Appendix*.

Effects of RNAi on Oviposition, Ovarian Development, and Hemolymph Reducing Sugar. After eclosion and dsRNA injection, females were separately kept in a plastic cup under the long-day condition. Seven days after eclosion, we placed two intact adult males with each female. From 7 to 21 d after eclosion, the number of eggs was counted at ZT2–4 every day. The hemolymph was collected 21 d after eclosion by using a pipette made from 1.0-mm outer-diameter glass capillaries (Narishige). Methods for measurement of the hemolymph reducing sugar level are described in *SI Appendix*. We examined the ovarian stage as described above.

OVX. Females were kept under the long-day condition after eclosion. One day (Day1 operation) or 10 d after eclosion (Day10 operation), we performed OVX or sham operations. Insects were placed on ice for a short time (~5 min), and we made a cut of ~1 mm with a razor in the abdomen near the ovary. The ovary was removed from the incision with tweezers. Subsequently, benzylpenicillin potassium (FUJIFILM Wako Pure Chemical Corporation) was applied to the incision to prevent bacterial infection. For the sham operation, we made the cut and applied the benzylpenicillin potassium to the incision without removing the ovary. Females were fasted for 2 d after surgery to recover. Twenty to 22 d after eclosion (19 to 21 [Day1 operation] or 10 to 12 d [Day10 operation] after the operations), we examined the ovarian stage and performed electrophysiological analyses.

Electrophysiology. We performed electrophysiological analyses in intact females, ds*bla*- and ds*per*-injected females, and sham- and OVX-operated females. Detailed methods are described in *SI Appendix*. Briefly, desheathed whole brains of *R. pedestris* were placed in the recording chamber filled with an extracellular solution containing basic ion components: 136 mM NaCl, 4.0 mM KCl, 10 mM Hepes, 2.0 mM $CaCl_2$, 1.5 mM $MgCl_2{\cdot}6\ H_2O$, and 10 mM glucose (pH was finally adjusted to ~7.4 with NaOH). Under an upright microscope, we approached the cell bodies of large neurons in the PI with a recording pipette. Then, we recorded the spontaneous firing activity in a whole-cell current-clamp mode. For whole-cell patch recording, we used a basic pipette solution containing 130 mM K^+-gluconate, 4.0 mM NaCl, 1.0 mM $MgCl_2{\cdot}6\ H_2O$, 0.5 mM $CaCl_2$, 10 mM ethylene glycol tetraacetic acid, and 10 mM Hepes (pH 7.2, adjusted with KOH). Additionally, we applied 20 mM neurobiotin tracer (Vector Laboratories) into the pipette solution to stain fiber projections from the recorded soma. The whole-cell patch recordings were filtered at 2 kHz with a multifunction filter 3611 (NF Corporation) and stored with an EPC7 patch-clamp amplifier (HEKA Elektronik), Digidata 1321A, Digidata 1550B (Molecular Devices), pCLAMP 8.2, and pCLAMP 11.0.3 software (Molecular Devices). All recordings were performed in the daytime (between ZT0 and ZT12), except the nighttime (ZT16–24) recording of intact on day 20–22 (*SI Appendix*, Fig. S3). Detection and analysis of the spontaneous spike activities were performed by using Clampfit 10.7 software (Molecular Devices).

Staining of Neuronal Projections from the Recorded Cell. To stain fiber projections from the recorded cell, we applied a positive current to inject the neurobiotion tracer into the recorded cell after electrophysiological recording. The whole brain was fixed in 4% paraformaldehyde (Chemical Abstracts Service No. 30525-89-4, TAAB) in 0.067 M phosphate buffer (PB; pH 7.4) overnight at 4 °C. After washing with 0.1 M phosphate-buffered saline with Triton X (PBST), the brain was incubated in 0.1 M PBST with an avidin–biotin complex (1:100; VECTASTAIN ABC Kit, product code PK-4001, Vector Laboratories) for 3 to 6 h at room temperature or overnight at 4 °C. The brain was washed with 0.1 M PBST and incubated in 0.1 M PBST with streptavidin Alexa Fluor 647 conjugate (1:200; product code S21374, Thermo Fisher Scientific Inc.) overnight at 4 °C. After washing with 0.1 M PBST, the brain was dehydrated by using an ethanol series and finally cleared by methyl salicylate (product code 139-03066, Wako).

Stained recorded cells were photographed and analyzed by using a Zeiss LSM710 confocal microscope with ZEN software (Carl Zeiss) and ImageJ software (NIH).

Single-Cell Reverse-Transcription Nested PCR. We prepared female *R. pedestris* 20 d after eclosion (labeled females a–e). The females were kept under short-day conditions (12-h light/12-h dark) at 25 ± 2 °C before eclosion and long-day conditions (16-h light/8-h dark) at 25 ± 2 °C after eclosion. We describe the single-cell PCR method in detail in *SI Appendix*. Briefly, in whole-brain preparations, we approached one large PI neuron with the pipette and isolated the cell by suction. The cell isolation was performed between ZT1 and ZT8. The isolated cell was immersed in reverse-transcription solution made by FastGene cDNA Synthesis 5x ReadyMix (Nippon Genetics), and cDNA was synthesized by TaKaRa PCR Thermal Cycler Dice (Takara Bio Inc.). Nested PCR was performed with a minor modification to the protocol of a previous study (61). After the PCR, we performed DNA electrophoresis and observed the bands on agarose gels. The primer sets for primary and secondary nested PCR for each gene are shown in *SI Appendix*, Table S4.

Statistical Analysis. Statistical analyses were performed with Kyplot5 software (KyensLab Inc.) using the Mann–Whitney *U* test, Tukey–Kramer test, Steel–Dwass test, and χ^2 test. Tukey-type multiple comparisons for proportions were carried out with Microsoft Excel 2016 (Microsoft Corporation). $P < 0.05$ was considered as statistically significant.

Data Availability. All relevant data are included in the manuscript and *SI Appendix*.

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