Roles of PER immunoreactive neurons in circadian rhythms and photoperiodism in the blow fly, *Protophormia terraenovae*

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SUMMARY

Several hypothetical models suggest that the circadian clock system is involved in the photoperiodic clock mechanisms in insects. However, there is no evidence for this at a neuronal level. In the present study, whether circadian clock neurons were involved in photoperiodism was examined by surgical ablation of small area in the brain and by immunocytochemical analysis in the blow fly *Protophormia terraenovae*. Five types of PER-immunoreactive cells, dorsal lateral neurons (LN$_d$), large ventral lateral neurons (l-LN$_v$), small ventral lateral neurons (s-LN$_v$), lateral dorsal neurons (DN$_l$) and medial dorsal neurons (DN$_m$) were found, corresponding to *period*-expressing neurons in *Drosophila melanogaster*. Four l-LN$_v$s and four s-LN$_v$s were bilaterally double-labelled with antisera against pigment-dispersing factor (PDF) and PER. When the anterior base of the medulla in the optic lobe, where PDF-immunoreactive somata (l-LN$_v$ and s-LN$_v$) are located, was bilaterally ablated, 55% of flies showed arrhythmic or obscure activity patterns under constant darkness. Percentages of flies exhibiting a rhythmic activity pattern decreased along with the number of small PDF-immunoreactive somata (i.e. s-Ln$_v$). When regions containing small PDF somata (s-LN$_v$) were bilaterally ablated, flies did not discriminate photoperiod, and diapause incidences were 48% under long-day and 55% under short-day conditions. The results suggest that circadian clock neurons, s-LN$_v$s, driving behavioural rhythms might also be involved in photoperiodism, and that circadian behavioural rhythms and photoperiodism share neural elements in their underlying mechanisms.

Key words: activity rhythm, diapause, lateral neurons, pigment-dispersing factor, photoperiodic response.

INTRODUCTION

For seasonal adaptation of life cycles, photoperiod is an important cue for plants and animals. Especially in insects, photoperiodic controls of seasonal phenotypes are widespread and observed in various developmental phases including reproduction, dormancy and migration. Although recent advances in understanding of physiological mechanisms underlying photoperiodism have been reported in plants and birds (Yasuo et al., 2006; Nakao et al., 2008; Turck et al., 2008), less knowledge is available in the case of insects. Insect photoperiodism has three physiological components: the photoreceptor, the photoperiodic clock and the effecter (Saunders, 2002). The photoperiodic clock entails a time-measurement system that usually measures the length of darkness per day (Saunders, 2002).

There have been several investigations of the physiological mechanisms underlying the photoperiodic clock in insects (Vaz Nunes and Saunders, 1999; Saunders, 2002), and it is generally accepted that circadian oscillators are involved in the time-measurement system (Vaz Nunes and Saunders, 1999; Veerman, 2001). To elucidate molecular or neural mechanisms underlying the photoperiodic clock, an understanding of circadian oscillator genes and neurons, and their relations with photoperiodism is needed. Based on the extensive knowledge of the circadian clock mechanism at the molecular level in *Drosophila melanogaster* (Stanewsky, 2003), several studies have tested the hypothesis that circadian clock genes function in the photoperiodic clock.

Two circadian clock genes have been examined for their roles in photoperiodism. Adult female *D. melanogaster* that have arrhythmic mutant alleles of the circadian clock gene *period* (*per^0*

mutants) show photoperiodic control of reproductive diapause, but the critical day length is less than in wild-type flies. This suggests that *per* is not causally involved (Saunders et al., 1989). In another drosophilid fly, *Chymomyza costata*, the circadian clock gene *timeless* has been shown to be crucial for photoperiodic control of larval diapause. In a non-photoperiodic-diapause (NPD) strain of *C. costata*, a single autosomal gene locus encoding *tim* was mutated, and both circadian eclosion rhythms and photoperiodic control of larval diapause were lost (Pavelka et al., 2003). The mutant lacking *tim* still enters diapause when exposed to low temperatures, suggesting that *tim* plays a role not in diapause induction, but in photoperiodic mechanisms (Riihimaa and Kimura, 1989). Recent studies on *D. melanogaster* have suggested that *tim* directly affects the incidence of diapause through circadian photoreception (Tauber et al., 2007; Sandrelli et al., 2007).

Although circadian clock genes have been the focus of studies on the relationship between the circadian clock and photoperiodic mechanisms, investigations of circadian clock neurons are also important. Insects have multiple circadian oscillator systems, and circadian clock genes are expressed in many cells in the whole body. In *D. melanogaster*, expression of *per* has been reported to oscillate throughout the body, and *per* in different tissues appears to drive different rhythms for various physiological phenomena (Plautz et al., 1997). Previous studies have shown the importance of the brain in photoperiodic mechanisms (Bowen et al., 1984); therefore, neurons expressing circadian clock genes in the brain should be investigated to elucidate photoperiodic mechanisms.

Since Helfrich-Förster (Helfrich-Förster, 1995) first characterized clock-gene-expressing neurons in the brain, understanding of

circadian oscillator mechanisms that result in complex behavioural rhythms has progressed in *D. melanogaster* (Stoleru et al., 2004; Grima et al., 2004; Rieger et al., 2006). In the *D. melanogaster* brain, six groups of neurons that express a set of circadian clock genes have been identified, and each neuron group appears to have a different role in behavioural rhythms (Helfrich-Förster et al., 2007; Grima et al., 2004; Stoleru et al., 2004; Rieger et al., 2006). Although in *D. melanogaster* circadian clock genes and neurons are well known, photoperiodic responses in this species are very shallow and difficult to assay. Clock-gene-expressing neurons or their protein-immunoreactive neurons have been examined in many species (Frisch et al., 1996; Sauman and Reppert, 1996; Závodská et al., 2005; Codd et al., 2007); however, their roles in behavioural rhythms or photoperiodism have not been identified. Only in the hawk moth *Manduca sexta*, has the loss of photoperiodic control of pupal diapause been shown after ablation of *per*-expressing neurons (Wise et al., 2002; Shiga et al., 2003). However, it is not known whether these neurons have roles in circadian rhythm oscillations in this species. Determining whether circadian clock neurons are a component for the photoperiodic clock would help identify photoperiodic clock neural networks and reveal integration mechanisms of photoperiodic information that may be active in the development of seasonal phenotypes.

Adult female blow fly *Protophormia terraenovae* (Robineau-Desvoidy 1830) (Diptera, Calliphoridae) show photoperiodic control of reproductive diapause. Females reproduce under long-day conditions, and enter diapause under short-day conditions (Numata and Shiga, 1995). The present study examined circadian clock neurons in the brain of *P. terraenovae*, and the effect of ablation of these neurons on photoperiodism. The results indicate that small ventral lateral neurons (s-LN$_v$s), which are immunoreactive to both Period (PER) and a neuropeptide, the pigment-dispersing factor (PDF), are prerequisites for circadian rhythm activity as in *D. melanogaster*. Furthermore, ablation of the s-LN$_v$s region resulted in a loss of photoperiodic discrimination. Involvement of s-LN$_v$s in photoperiodism and a plausible neural network for photoperiodic control of diapause are discussed.

MATERIALS AND METHODS
Insects and experimental schedules

P. terraenovae were kept as a laboratory culture under long-day conditions (18 h:6 h L:D, at 25±1°C) to avoid diapause. Larvae were fed beef liver, and adults fed sucrose, beef liver and water as in the study by Numata and Shiga (Numata and Shiga, 1995). For PER immunocytochemistry analysis and for recording activity rhythms, insects were reared under 12 h:12 h L:D at 20±1°C. On days 6–11 (6 to 11 days after adult emergence), adult females were used for immunocytochemical analysis. For the photoperiodism experiments, insects were reared under 12 h:12 h L:D at 25°C. Ten to 15 females were collected on day 0, and held under 18 h:6 h L:D or 12 h:12 h L:D at 25°C. During days 1–14, the females were fed sucrose and water. Beef liver, which is necessary for ovarian development, was given for the last 3 days, and ovarian development was examined on day 14. Ovarian stages were determined according to Matsuo et al. (Matsuo et al., 1997). Flies with previtellogenic ovaries at stages 1 and 2– were considered in diapause, whereas those with vitellogenic ovaries at stages 2+ to 6 were deemed reproductive (i.e. non-diapause).

Immunocytochemistry

The heads of female flies were cut off at zeitgeber time 0–1 (ZT 0–1; 0–1 h after light-on) and the posterior cuticle removed to expose the brain to fixatives. The head was fixed in 4% paraformaldehyde for 4 h at 4°C for whole-mount preparations, or in aqueous Bouin's fixative overnight at room temperature for paraffin sections. PER immunocytochemistry was performed using the ABC method (Vectastain ABC standard kit; Vector Laboratories, Burlingame, CA, USA).

For staining of brain whole mounts, the brain was excised and washed in phosphate-buffered saline (PBS) with 0.5% Triton X-100 (PBST) overnight with several changes of PBST. The brain was incubated in 0.3% H$_2$O$_2$ for 1 h at room temperature to reduce endogenous peroxidase activity and in 0.5% BSA for 1 h. Then, goat anti-*D. melanogaster*-PER antiserum (sc-15720; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added at a working dilution of 1:1000. This polyclonal antibody was raised against a peptide mapping at the N terminus of PER of *D. melanogaster* origin. Brains were kept in the primary goat antiserum for 3 days at 4°C. This was followed by incubation in a secondary antiserum, donkey anti-goat immunoglobulin conjugated with biotin (705-065-003; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), at a dilution of 1:200 for 1 day at 4°C. Both primary and secondary antisera were diluted in PBST containing 0.5% bovine serum albumin. Whole brains were incubated in an avidin–biotin complex solution at a dilution of 1:100 for 1 day at 4°C. After washing in PBST, the brains were preincubated in diaminobenzidine (Sigma, St Louis, MO, USA) for 1 h at 4°C, and incubated in a mixture of 0.01% H$_2$O$_2$ and 0.03% diaminobenzidine for 15–30 min at room temperature. After washing with PBST, whole-mount preparations were dehydrated in an ethanol series, and cleared in methyl salicylate for observation.

Double labelling with anti-PER antiserum (ABC method) and anti-PDF antiserum (fluorescence immunocytochemistry) was performed on paraffin sections. Paraffin sections (8 µm thick) were made using a standard protocol. After thoroughly removing the paraffin, sections were incubated in 0.3% H$_2$O$_2$ for 30 min at room temperature. Subsequently, they were incubated in PBS with 0.5% BSA for 20 min at room temperature and then in the primary PER antiserum (1:1000) overnight at 4°C. After washing with PBS, the primary antiserum-treated sections were incubated in the secondary antiserum conjugated with biotin for 1 h, and then in an avidin–biotin complex solution for 1 h at room temperature. After washing with PBS, sections were incubated in a mixture of 0.01% H$_2$O$_2$ and 0.03% diaminobenzidine for 2–7 min at room temperature. Then, sections were washed thoroughly in PBS, and the PER-stained sections were processed for PDF immunocytochemistry. The rabbit anti-*Gryllus bimaculatus*-PDF antiserum was provided by Dr K. Tomioka (Okayama University, Okayama, Japan). The epitope structures recognized by the anti-*Gryllus bimaculatus*-PDF have been well characterized by enzyme-linked immunosorbent assay (Honda et al., 2006). The sections were incubated in the anti-PDF antiserum at a dilution of 1:5000 for 1 day at 4°C. Swine anti-rabbit immunoglobulin antiserum conjugated with tetramethylrhodamine isomer R (R0156; Dako, Glostrup, Denmark) was used as a secondary antiserum at a dilution of 1:200 for 1 day at 4°C. After washing thoroughly in PBS, sections were dehydrated and mounted in methyl salicylate.

A specificity test was performed using a pre-adsorption technique. The anti-PER antiserum at a dilution of 1:1000 was incubated in 200 µg ml^{-1} of Period protein (sc-15720 P; Santa Cruz Biotechnology) overnight at 4°C. Immunocytochemistry was performed on whole-mount preparations using the antigen–antiserum complex instead of the primary antiserum. No staining was observed in this control experiment (data not shown).

In brains subjected to surgical operation, PDF immunocytochemistry was undertaken after recording activity rhythms or ovarian stages. Whole-mount brains were processed for PDF immunocytochemistry using the above mentioned ABC method. A dilution of 1:5000 of the rabbit anti-PDF antiserum was used as the primary antiserum.

Images were studied with a compound photomicroscope (BX50-33DIC, Olympus, Tokyo, Japan) or an epifluorescence microscope (BX50-34FLA-3; Olympus). Double-labelled neurons were viewed as a bright-field image for diaminobenzidine, and the exactly the same area at the same depth was viewed as a fluorescence image for tetramethylrhodamine. The images were digitalized with a CCD camera (CoolSNAP; Nippon Roper, Chiba, Japan) and processed using Adobe Photoshop 6.0 (Adobe System Incorporated, Tokyo, Japan) for colour adjustment to the whole image if necessary, and by Corel Draw 9.0 (Corel, Ottawa, ON, Canada) for lettering.

Surgical operations

Surgical removal of PDF-immunoreactive (PDF-ir) neurons in the optic lobe was performed to examine their roles in circadian rhythms and photoperiodism. For examination of their role in activity rhythms, regions including large and small PDF somata were ablated bilaterally (site 2 in Fig. 1A). Because rhythm deficiency was detected when small PDF somata were lost, regions smaller than those shown in site 2 (site 4 in Fig. 1B) were ablated for examination of their role in photoperiodism. Day-1 females were mounted in clay with the frontal face exposed and then placed on ice for 10–30 min. Subsequently, under a stereomicroscope a small pool of 0.9% NaCl was placed on the fly's face and bilateral vertical incisions were made along the medial edge of each compound eye with bilateral horizontal incisions above the antennae. The frontal face of the head cuticle was then opened to expose the anterior brain surface. For examination of role of PDF-ir neurons in circadian activity rhythms, portions of the anterior base of the medulla (site 2 for the test group) or of the anterior-lateral region of the protocerebrum (site 1 for the control-operated group) were bilaterally ablated with a sharpened tungsten needle under 0.9% NaCl solution (Fig. 1A). Following the operation, the cuticle was returned to its original position. In a sham operation, females were processed as above; however, no brain portions were removed. To examine the role of small PDF-ir neurons in photoperiodism, smaller portions of site 4 were removed (Fig. 1B). As a control, the anterior dorsal part of the protocerebrum was bilaterally ablated (site 3 operation; Fig. 1). A sham-operated group was also prepared.

Recording and analysis of locomotor activity rhythms

The activity recording system was adopted from Hamasaka et al. (Hamasaka et al., 2002). Locomotor activity was recorded as the number of times that the fly interrupted the infrared beam (EE-SPW321, Omron, Kyoto, Japan). The number of events, summed every 6 min, was collected by a Microsoft Windows-based personal computer. Following surgical operation, adult females on day 1 were individually placed in the recording chamber. Activity was recorded under constant darkness (DD) at 20°C for 7–8 days and subsequently under 12 h:12 h L:D or 18 h:6 h L:D at 25°C for 10 days. The light intensity ($1.4\,W\,m^{-2}$) was provided by a white fluorescent lamp (FL15W; National, Osaka, Japan) during the photophase. Rhythmicity was evaluated by a χ^2 periodogram (Sokolove and Bushell, 1978). To analyse diel activity levels, relative values of events every 30 min were calculated each day, and daily means of the relative values were calculated for each fly. Average values of the daily means were calculate, and were plotted as relative activity levels for each experimental group (see Fig. 6).

RESULTS
Period-immunoreactive neurons

A homolog of the *period* gene has been partially cloned in adults *P. terraenovae*, and a 721 bp fragment shows 65% similarity to *period* in *D. melanogaster* (Goto and Numata, 2003). We used an antibody against *D. melanogaster* PER in our experiments. In whole-mount preparations, three cell clusters in the boundary between the optic lobe and mid-brain, and two clusters in the protocerebrum were distinctively labelled (Figs 2 and 3). After the nomenclature used in *D. melanogaster*, cell clusters at the boundary were named dorsal lateral neurons (LN_ds), large-type ventral lateral neurons (l-LN_vs) and small-type ventral lateral neurons (s-LN_vs) (Helfrich-Förster, 1995; Kaneko and Hall, 2000). In the dorsal protocerebrum, two groups of dorsal neurons (DNs) were found. We designated a cluster in the medial region as medial dorsal neurons (DN_ms) and a cluster in the lateral region as lateral dorsal neurons (DN_ls). In comparison with *D. melanogaster*, *Protophormia* DN_ms seem to correspond to *Drosophila* DN_1s and DN_2s and *Protophormia* DN_ls correspond to *Drosophila* DN_3s.

In the anterior boundary between the optic lobe and protocerebrum, five LN_ds with a diameter of 7.1±1.0 μm (mean ± s.d., $N=9$) were dorsally located, and four l-LN_vs with a diameter of 14.2±3.7 μm ($N=8$) and five s-LN_vs with a diameter of 7.1±1.3 μm ($N=15$) were ventrally located (Fig. 2A,B). The l-LN_vs were located dorsally to the s-LN_vs (Fig. 2A). In the dorsal posterior region of the protocerebrum, 11–14 cells were classified as DN_m (7.1±1.2 μm, $N=37$) and four to six DN_l (7.0±1.5 μm, $N=16$), respectively (Fig. 2C). DN_ms were found in the pars lateralis (PL), in which neurosecretory cells are located. At ZT 0–1, stronger immunoreactivity was present in the nucleus than in the cytoplasm (Fig. 2A).

Double labelling with anti-PER and anti-PDF antisera was carried out (Fig. 4) because the cell locations of l-LN_v, s-LN_v and
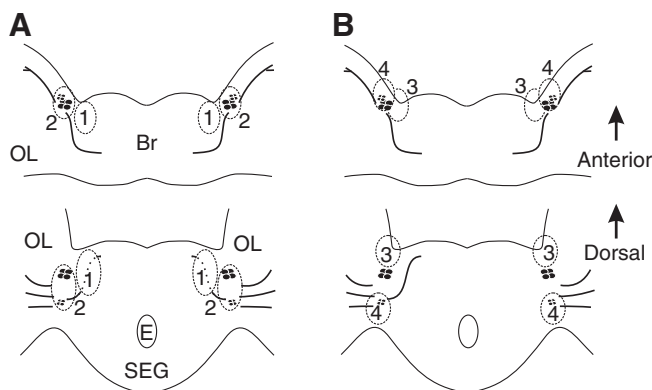

Fig. 1. Schematic illustrations of ablated regions in the adult brain of *Protophormia terraenovae*. Four large pigment-dispersing factor (PDF)-immunoreactive somata (large filled circles), four small PDF-immunoreactive somata (small filled circles), and their fibres are shown bilaterally in dorsal (upper) and frontal (lower) views. Ablated regions are encircled with dotted lines. (A) For examination of activity rhythms, site 1 (control) and site 2 (test) were ablated (site 1 and site 2 operations, respectively). Note that site 1 is located anterior to the PDF-immunoreactive fibres and does not include those fibres. (B) For examination of photoperiodism, site 3 (control) and site 4 (test) were ablated (site 3 and site 4 operations, respectively). Br, brain; E, oesophageal foramen; OL, optic lobe; SEG, suboesophageal ganglion.
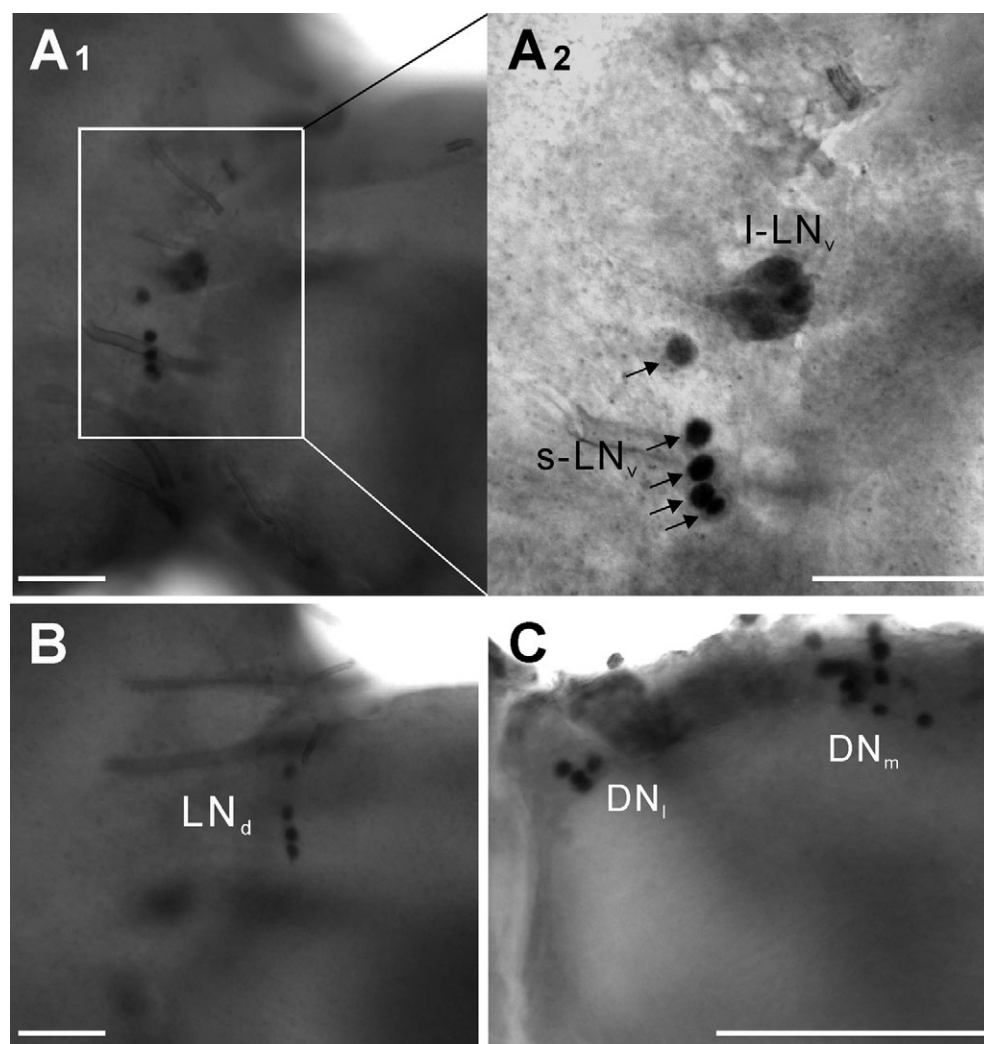
Fig. 2. Photomicrographs of PER-immunoreactive cells in the adult brain of *Protophormia terraenovae* (whole-mounts, frontal views, dorsal region to the top and medial to the right). (A) In an anterior ventral boundary between the left optic lobe and mid-brain, a cluster of four large ventral lateral neurons (l-LN$_v$s) and five small ventral lateral neurons (s-LN$_v$s) are shown. A2 is an enlarged image of the boxed region in A1. Arrows indicate five s-LN$_v$s. (B) In an anterior dorsal boundary between the left optic lobe and mid-brain, four dorsal lateral neurons (LN$_d$s) are in focus. (C) The dorsal posterior region of the protocerebrum, showing a stained cluster of lateral dorsal neurons (DN$_l$s) and a stained cluster of medial dorsal neurons (DN$_m$s). Only part of DN$_l$s and DN$_m$s are in focus. Scale bars, 100 μm.

DN$_m$ were quite similar to that of PDF-ir somata in *P. terraenovae* (Nässel et al., 1993; Hamanaka et al., 2007). All four l-LN$_v$s and four of five s-LN$_v$s were also immunolabelled with PDF antiserum. Four large and four small PDF-ir neurons with somata at the anterior base of the medulla were l-LN$_v$s and s-LN$_v$s, respectively (Fig. 4A,B). In the posterior dorsal protocerebrum, DN$_m$s appeared in close proximity to the PDF somata. There were eight PDF somata in the pars lateralis. Of these, three are reported to extend axons into the retrocerebral complex to innervate the corpus cardiacum and hypocerebral ganglion, and five are local neurons (Hamanaka et al., 2007). PER immunoreactivity mainly occurred in different cells from PDF somata in the pars lateralis. However, at least one cell seemed to be labelled by both PER and PDF antisera (Fig. 4C). In most sections, the PER antiserum labelled both the nuclear and cytoplasmic regions, whereas the PDF antiserum mainly labelled cytoplasmic areas (Fig. 4). Although the PDF antiserum stained axons and fibres along with somata, the PER antiserum stained only somata.

Effects of removal of PDF-immunoreactive neurons on circadian activity rhythm

Activity under DD was classified into three patterns: rhythmic, obscure and arrhythmic, and their incidences were compared (Fig. 5). In the obscure pattern, activity was classified neither as rhythmic nor typically arrhythmic under DD (Fig. 5D). Most intact

and all sham-operated flies free-ran with a period of 24.9±0.6 h (mean ± s.d., *N*=31) in intact and 24.9±0.9 h (*N*=14) in sham-operated flies under DD (rhythmic pattern), and were entrained to short-day and long-day cycles with activities in the photophase (Fig. 5A, Fig. 6). In both short- and long-day cycles, activities continued during the photophase irrespective of the photophase length (Fig. 6).

Among flies operated on at site 1, in which a small region of the anterior lateral protocerebrum was ablated (*N*=11; Fig. 1A), 10 out of 11 females showed rhythmic patterns with free-running periods of 25.0±1.0 h (*N*=10; Fig. 5B). In one female, the obscure pattern was observed. Under LD cycles, most females were entrained to the cycles. Some females, however, continued to be active for several hours even after light-off, or exhibited longer transient periods. In these females, even though activities decreased at the end of the photophase, high average activity levels were observed at the first half of the scotophase compared with the intact or sham-operated groups (Fig. 6). For histological examination of ablated regions, PDF immunocytochemistry was performed (Fig. 7). PER immunocytochemistry only stained somata; therefore, PDF antiserum, which stains both somata and fibres of l-LN$_v$s and s-LN$_v$s, was used. PDF-ir neurons were successfully stained in eight of 11 females with site 1 operations. All four large and four small PDF-ir neurons in the optic lobe were bilaterally present in seven of eight females. The female with the obscure pattern also contained
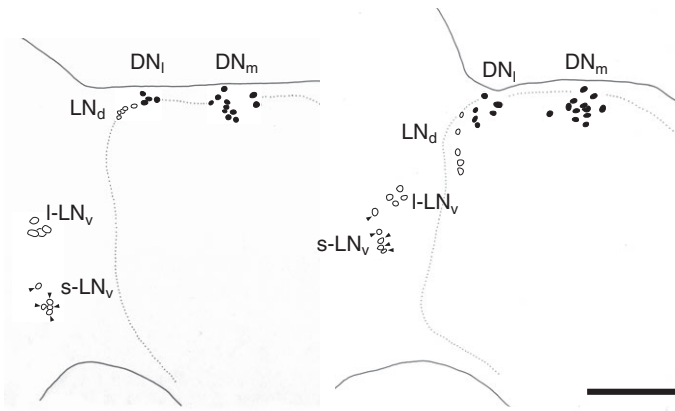
Fig. 3. Tracings of PER-immunoreactive cells in a left hemisphere in two representative adult brains of *Protophormia terraenovae*. See legend of Fig. 2 for abbreviations. Open and filled circles indicate cells located in anterior and posterior regions, respectively. Scale bar, 100 μm.

all PDF-ir neurons. In one female, two large PDF-ir neurons were missing in one optic lobe, but its activity showed a rhythmic pattern with entrainment to LD cycles. In all site 1-operated females, PDF-ir fibres were stained as observed in intact brains.

When the anterior base of the medulla was bilaterally ablated (N=31, site 2 operation) (Fig. 1A), 25.8% of the flies showed arrhythmicity and 29.0% showed the obscure pattern under DD (Fig. 5C,D, Fig. 8A). The remaining flies were rhythmic with a free-running period of 24.6±1.0 h (N=14) under DD. In the obscure pattern group, some females showed rhythmic activity for a few days immediately after surgery, followed by arrhythmia (Fig. 5D). The rhythmic, obscure, or arrhythmic activities observed under DD continued under LD conditions in most females. Some females showing rhythmic patterns under DD became arrhythmic under LD. No females with the arrhythmic pattern under DD were subsequently rhythmic under LD. After the site 2 operation, masking effects, in which activities in the scotophase are higher than those in the photophase, were observed in some flies both under long- or short-day LD cycles (Fig. 5D, Fig. 6). The masking effects under LD
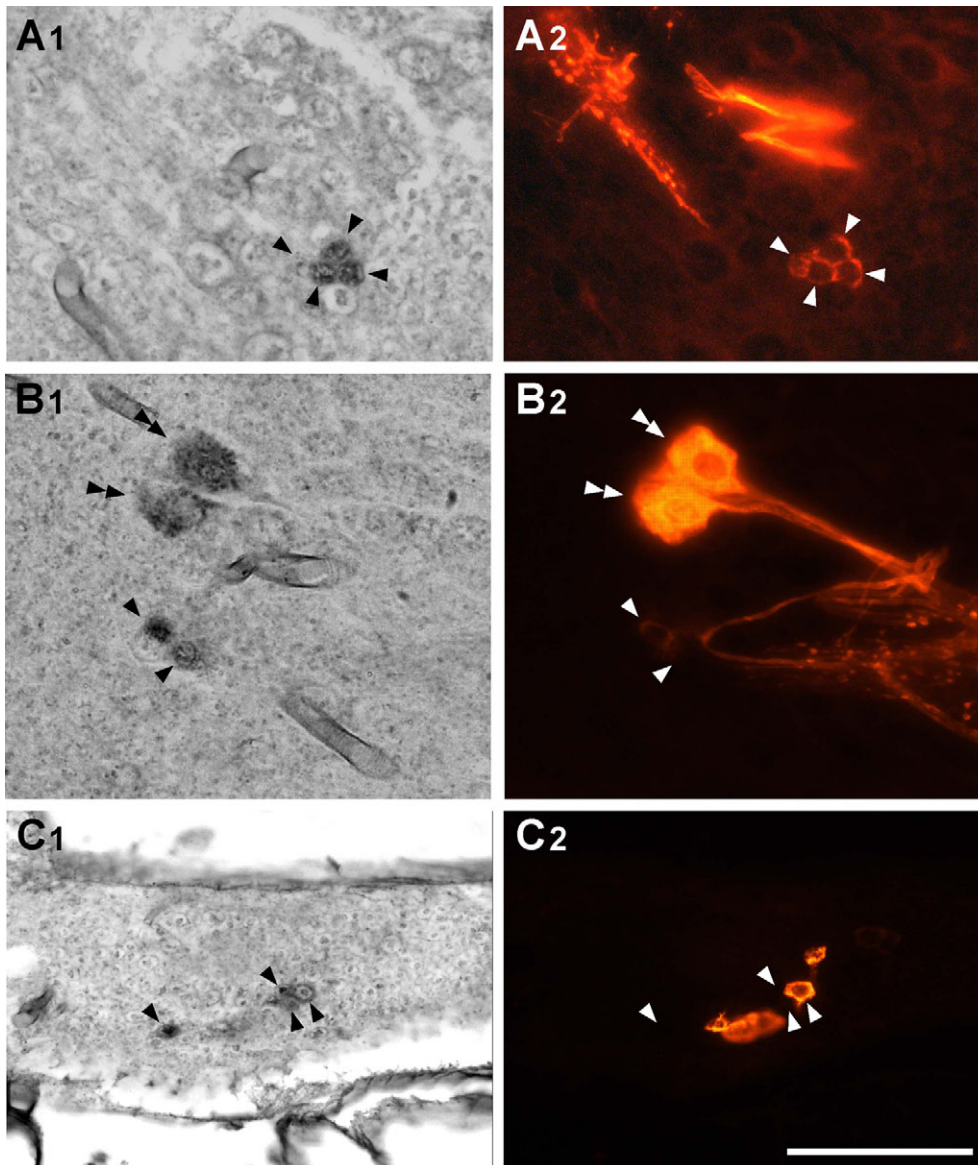

Fig. 4. Photomicrographs of neurons labelled by PER and pigment-dispersing factor (PDF) antisera in the adult brain of *Protophormia terraenovae*. Stained neurons were viewed as bright-field images for PER-immunoreactive cells (A1, B1 and C1) and the exactly same areas at the same depth was examined as fluorescent images for PDF-immunoreactive neurons (A2, B2 and C2). Note that the cytoplasm and fibres were labelled using the PDF antiserum, whereas the PER antiserum labelled only somata. Arrowheads are located in exactly the same positions in the respective right and left panels. (A) Four s-LNvs (single arrowheads). (B) Two l-LNvs (double arrowheads) and two s-LNvs (single arrowhead). Note that one s-LNv is not labelled by the PDF antiserum. (C) Four DNms (single arrowheads) are present, but only one DNm is labelled by the PDF antiserum. Scale bar, 50 μm.
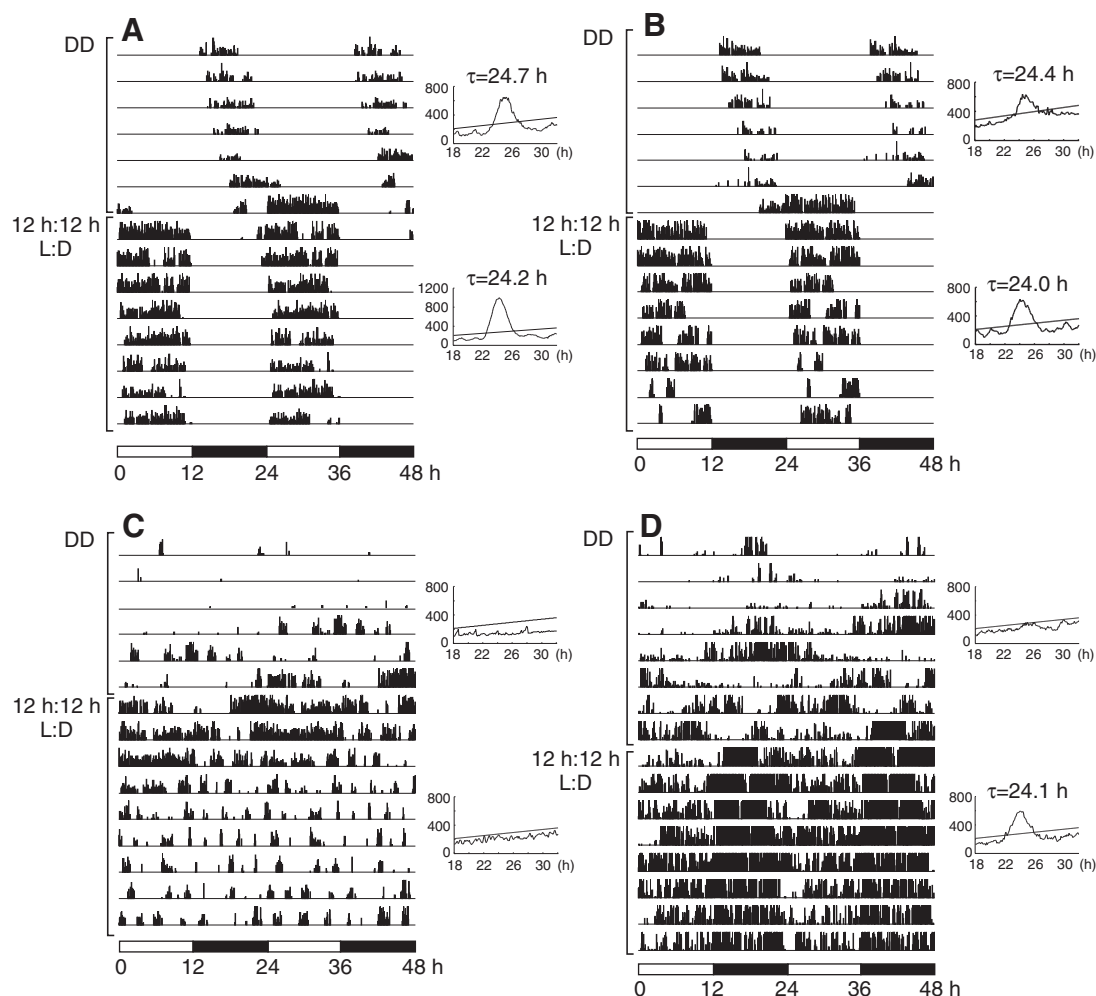
Fig. 5. Locomotor activity rhythms in flies after sham operation (A), site 1 operation (B) and site 2 operation (C,D) in adult *Protophormia terraenovae*. Locomotor activities were recorded under constant darkness (DD) for 7–8 days and under 12 h:12 h L:D for 9 days. Activities are shown in double plotted actograms with associated periodogram analysis results. White and black bars at the bottom of actograms indicate respective light and dark periods. Flies after sham or site 1 operations showed rhythmic patterns (A,B), but arrhythmic (C) and obscure patterns (D) appeared after site 2 operations.

conditions occurred irrespective of the activity patterns under DD. In the site 2-operated group, average activity levels in the scotophase were almost equal to or higher than those in the photophase (Fig. 6).

Fig. 7 shows tracings of PDF-ir neurons in the intact and site 2-operated groups. After the site 2 operation, different numbers of PDF-ir neurons in the optic lobe remained. The number of remaining PDF somata was counted in each fly, and the flies were then grouped according to these numbers, and incidences of the three activity patterns were calculated for each group (Fig. 8B). When flies were sorted according to the number of large PDF somata, no correlations were found between incidence of the rhythmic pattern and the number of large PDF somata (Fig. 8B, left). However, when the same data were sorted according to the number of small PDF somata, rhythmic pattern incidences depended on the number of somata (Fig. 8B, right). The data show that the fewer small PDF-ir neurons that remained, the fewer flies that showed the rhythmic pattern. In females from which all large PDF somata were removed (*N*=7; Fig. 8B, left), two, three and two females showed arrhythmic, obscure and rhythmic pattern, respectively. Both of the arrhythmic females had no small PDF somata. The numbers of small PDF somata in the three females exhibiting the obscure pattern were four, zero and zero. The number of small PDF somata in the two females

showing rhythmic activity was one and seven. Even in the complete absence of large PDF somata, rhythmic activity occurred, if some small PDF somata were present.

Effects of removal of PDF-immunoreactive neurons on photoperiodism

The activity pattern results suggested that small PDF-ir neurons are important for generating an activity rhythm under DD. Therefore, brain regions containing small PDF somata were bilaterally ablated to examine the effects on photoperiodism (site 4 operation; Fig. 1B). As a control, the anterior dorsal boundary between the optic lobe and mid-brain was bilaterally ablated (site 3 operation; Fig. 1B). In the intact, sham-operated and control-operated groups, most flies entered diapause under short-day conditions, whereas under long-day conditions most flies were reproductive (Fig. 9). Diapause incidences in intact, sham-operated, and control-operated groups under short-days were significantly higher than those under long-days (*P*<0.05, Tukey type multiple comparison for proportions) (Zar, 1999) (Fig. 9A). In the site 4-operated group, diapause incidence was 55.1% (*N*=49) under short-day and 48.4% (*N*=31) under long-day conditions, and no significant difference was detected (*P*>0.05, Tukey type multiple comparison for proportions; Fig. 9A).
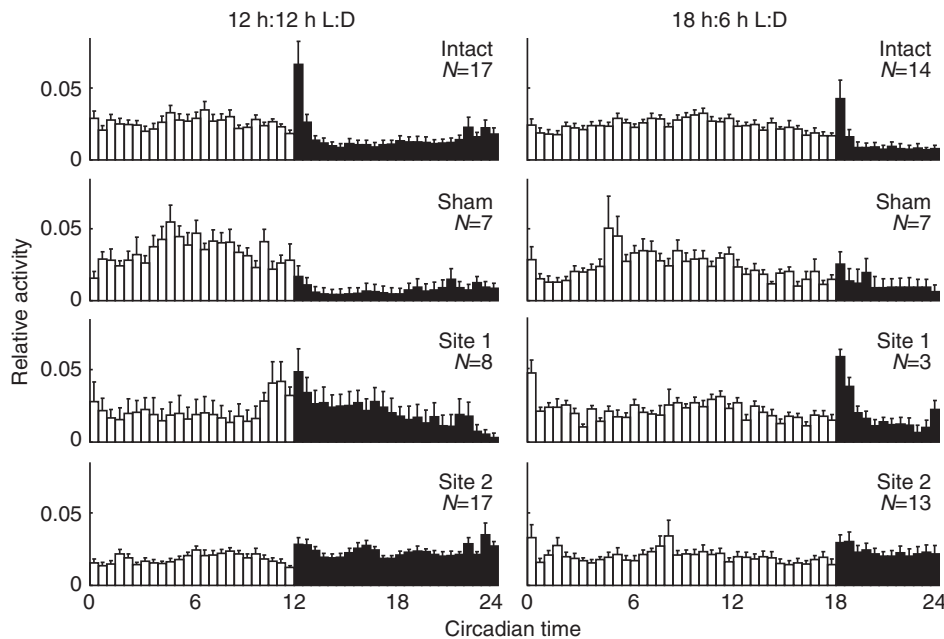
Fig. 6. Diel activity levels in intact, sham-operated, site 1-operated and site 2-operated adult *Protophormia terraenovae* under 12 h:12 h and 18 h:6 h L:D. Relative activity levels were generated as described in the text. Each column corresponds to a 30-min period. The open columns represent relative activity levels during the photophase, and the filled columns those during the scotophase. Bars above each column show s.e.m. (i.e. fly-to-fly variation in relative activity level for a given period).

After examination of ovarian stages, brains were subjected to PDF immunocytochemistry. In the control-operated group (site 3 operation) PDF-ir neurons were successfully stained in all females (*N*=12) under short-day conditions and in 13 of 14 females under long-day conditions, whereas in the site 4-operated group, 46 of 49 females under short-day and 30 of 31 females under long-day conditions showed successful staining. In the control-operated group, all PDF somata (four pairs of large somata and four pairs of small somata) remained in most females. In most site 4-operated females, half or more of the large PDF somata remained, and half or more of the small PDF somata were ablated. No correlations were detected between the remaining number of large PDF somata and diapause phenotypes (data not shown). This was also the case for the number of small PDF somata (Fig. 9B). Even in females in which all the small PDF somata were ablated, both diapause and

non-diapause females were observed (Fig. 9B). In those females with no small PDF somata remaining, under short-day conditions the number of large PDF somata was two to eight in both diapause (*N*=10) and non-diapause females (*N*=9), and under long-day conditions there were five to eight large PDF somata in diapause (*N*=6) and four to eight in non-diapause females (*N*=3). Although no significant correlations between the remaining number of PDF somata and diapause incidences were detected after site 4 operations, the effect of ablation of site 4 was different from that of site 3. This suggests that site 4 is important for photoperiodic control of diapause.

DISCUSSION

per is an important circadian clock gene in *D. melanogaster* (Konopka and Benzer, 1971; Baylies et al., 1987), and circadian oscillation of
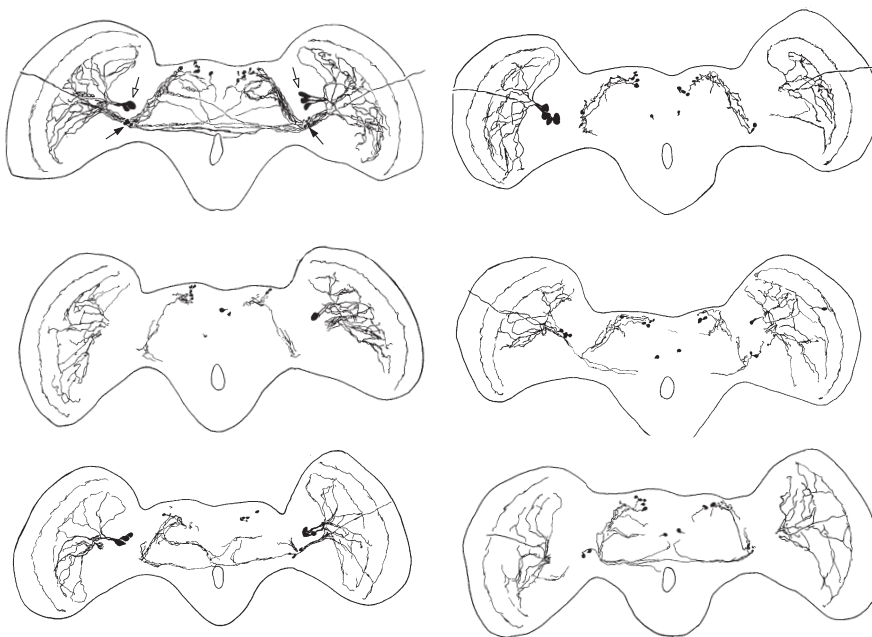

Fig. 7. Representative tracings of PDF-immunoreactive neurons in the adult brains of *Protophormia terraenovae*. Top left is an example of the intact group and the others are five of the site 2-operated group. In the intact brain, four small (black arrows) and four large PDF somata (white arrows) were bilaterally stained. In the site 2-operated examples different numbers of PDF somata remained. Scale bar, 100 μm.
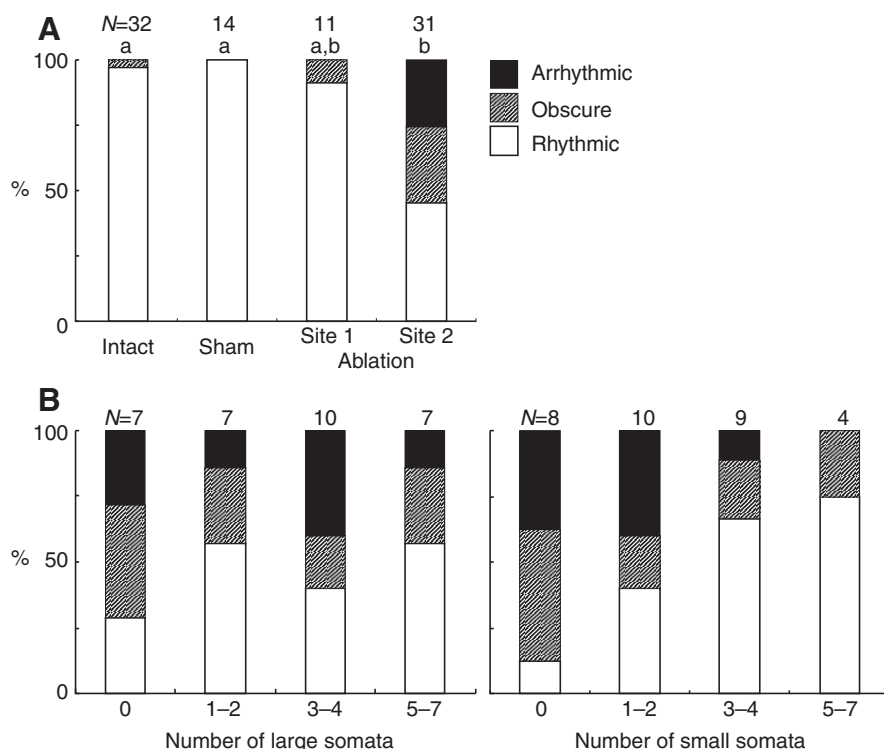
Fig. 8. Effects of the surgical operation on rhythmicity under constant darkness in adult *Protophormia terraenovae*. (A) In intact, sham-operated and site 1-operated (control) groups, most females showed rhythmic patterns. After a site 2 operation, however, locomotor activities were arrhythmic in 25.8% and obscure in 29% of flies. Different letters above the columns indicate significant differences in incidences of females with rhythmic patterns (Tukey-type multiple comparison test for proportions, *P*<0.05). (B) Results after site 2 operations are shown according to the number of remaining PDF somata. The number of PDF somata was counted in each fly, and flies were grouped according to the number of large PDF somata (left) and small PDF somata (right). There were no flies in which all large (*N*=8) or all small PDF somata (*N*=8) remained. Incidences of the rhythmic patterns depended on the number of remaining small PDF somata; the fewer small PDF somata remaining, the fewer flies that showed rhythmic patterns.

per mRNA or distribution of *per*-expressing neurons has been shown in different insect orders (Frisch et al., 1996; Sauman and Reppert, 1996; Goto and Denlinger, 2002; Wise et al., 2002; Hodková et al., 2003; Závodská et al., 2005; Iwai et al., 2006; Codd et al., 2007; Moriyama et al., 2008). However, few studies have demonstrated that *per* or *per*-expressing neurons are functionally important for circadian behavioural rhythms or photoperiodism. In *G. bimaculatus*, it has been reported that *per* knockdown by RNAi suppresses circadian rhythms in locomotor activities and electrical activities of optic lobe neurons (Moriyama et al., 2008). In *M. sexta*, surgical ablation of *per* expressing neurons from larval brains caused loss of photoperiodic control of pupal diapause (Wise et al., 2002; Shiga et al., 2003). Although these studies suggest the importance of *per* or *per*-expressing neurons in clock mechanisms, there have been no reports on how these neurons integrate or process timing information in the brain. The present study detected five groups of PER-ir neurons in the brain of *P. terraenovae* and the results suggest that PER-ir s-LN$_v$s are important for driving circadian activity rhythms and the site 4 containing s-LN$_v$s for photoperiodism. These results, together with the results of our previous studies, suggest a plausible neural network for photoperiodic control of diapause.

Distribution of PER-immunoreactive neurons

In *P. terraenovae*, distribution of PER-ir cells in the brain and colocalization patterns of PER with PDF were similar to that reported in *D. melanogaster* (Helfrich-Förster, 2003). Also five s-LN$_v$s, four of which were PDF-immunopositive were shown to be present in *P. terraenovae* as in *D. melanogaster*. Only one PDF-immunonegative s-LN$_v$ is present in *P. terraenovae* and *D. melanogaster*, whereas four PDF-immunonegative s-LN$_v$s were found in the house fly *Musca domestica* (Codd et al., 2007). However, the numbers of l-LN$_v$s and LN$_d$s were similar in *D. melanogaster*, *M. domestica* and *P. terraenovae*.

In the dorsal protocerebrum, DN$_1$ in *P. terraenovae* seems to correspond to DN$_3$ in *D. melanogaster*. However, only four to six

DN$_l$s were stained, which are much fewer than the DN$_3$s (up to 40 cells) in *D. melanogaster*. In the medio-dorsal protocerebrum of *D. melanogaster*, two groups, DN$_1$ and DN$_2$, have been identified, with different fibre projections (Kaneko and Hall, 2000). *P. terraenovae* may also have two clusters in DN$_m$, but discrimination of the two is difficult without identification of fibre projections. Also fewer DN clusters have been reported in *M. domestica* than in *D. melanogaster* (Codd et al., 2007). Although there are some differences from *D. melanogaster*, it seems that *P. terraenovae* retains a homologous set of PER-ir neurons, as does *M. domestica*. These neural networks might be common to all the dipteran Cyclorrhapha.

In *P. terraenovae*, nuclear staining at ZT 0–1 suggests that PER protein enters the nucleus and there is a possibility that PER plays some role in regulation of clock gene expressions, as in *D. melanogaster* (Curtin et al., 1995; Shafer et al., 2004). Analysis of other circadian clock genes is required in future studies.

s-LN$_v$s are important for circadian activity rhythms

Molecular tools are not available for *P. terraenovae*; therefore, the roles of PER-ir neurons in circadian activity rhythms and photoperiodism were examined by microsurgery. We focused on LN$_v$s as their importance for activity rhythms under DD was shown in *D. melanogaster* (Helfrich-Förster, 2003). The incidence of rhythmic patterns and the number of small PDF somata (s-LN$_v$s) remaining after surgery correlated, whereas no correlations were observed between the number of large PDF somata (l-LN$_v$s) and activity patterns (Fig. 8B). The results suggest that s-LN$_v$s are required to drive locomotor activity rhythms under DD. Only one fly showed rhythmicity when all small PDF somata were missing. This might be caused by other *per*-expressing neurons. It is suggested in *D. melanogaster* that PDF-negative lateral neurons (LNds and one sLNv) or a subset of DNs control rhythmic behaviour under constant light conditions (Rieger et al., 2006; Murad et al., 2007; Picot et al., 2007). If it is also true in *P. terraenovae* then
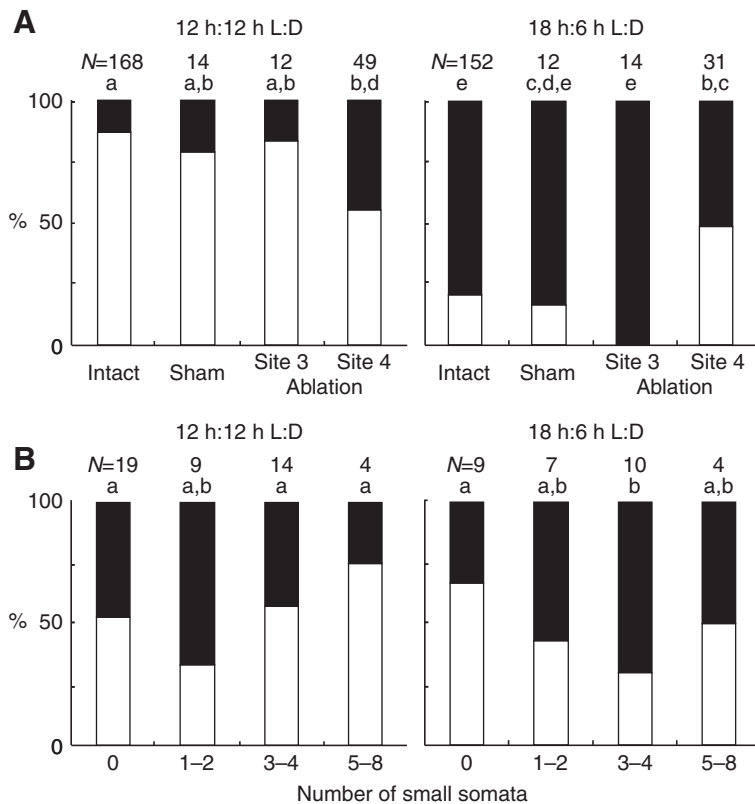
Fig. 9. Effects of the surgical operation on photoperiodism in adult *Protophormia terraenovae*. (A) In intact, sham-operated, and site 3-operated groups, most females entered diapause under 12 h:12 h L:D, whereas most females developed ovaries under 18 h:6 h L:D. After site 4 operation, flies did not show photoperiodism, and diapause incidences were 55.1% under 12 h:12 h L:D and 48.4% under 18 h:6 h L:D. (B) Results after site 4 operation are shown according to the number of remaining small PDF somata. Filled and open columns show reproductive females and females in diapause, respectively. Different letters above the columns indicate significant differences in incidences of females in diapause (Tukey-type multiple comparison test for proportions, $P<0.05$).

these neurons might drive the activity rhythm even under DD if the cellular networks were affected by the surgery.

Compared with DNs, the importance of LNs as pacemaker neurons for activity rhythms has been shown in *D. melanogaster* (Frisch et al., 1994; Helfrich-Förster, 1998; Grima et al., 2004; Stoleru et al., 2004), and the dominant roles of s-LN$_v$s in driving circadian activity rhythms have been reported (Stoleru et al., 2005; Helfrich-Förster et al., 2007). Our results in *P. terraenovae* support those observations. Because LN$_d$s are located far dorsal to the s-LN$_v$s, it is unlikely that s-LN$_v$s ablation invaded the LN$_d$s regions. We suggest, therefore, that LN$_d$s are not capable of driving activity rhythms without the presence of s-LN$_v$s under DD. Although the possibility that LN$_d$s plays a role in activity rhythms cannot be excluded, we consider LN$_d$s to play a less dominant role than s-LN$_v$s under DD.

After removal of the anterior base of the medulla, residual rhythms were observed for a few days in some flies (Fig. 5D). Similar patterns have been reported in behaviourally arrhythmic *disconnected* mutant *D. melanogaster* in which PDF-ir neurons are missing (Helfrich-Förster and Homberg, 1993; Wheeler et al., 1993; Helfrich-Förster, 1998). Residual rhythms in *disconnected* flies appeared for several days under DD, although *per0* mutant flies are completely arrhythmic under DD (Wheeler et al., 1993). Such residual rhythms could be explained by the presence of other *per*-expressing neurons.

P. terraenovae exhibits diurnal locomotor activity rhythms (Hamasaka et al., 2002). After the site 2 operations, however, some flies were nocturnally active under LD conditions, irrespective of their activity pattern under DD. In *D. melanogaster* the importance of l-LN$_v$s in light-arousal activities by altering electrical activities of the LN$_v$ has been reported just recently (Sheeba et al., 2008; Shang et al., 2008). *D. melanogaster* shows bimodal rhythms with morning and evening activities. When LN$_v$s were hyper-excited, enhancement of the nocturnal locomotor activity was observed and the normal

day–night firing patterns of the action potentials in l-LN$_v$s were reversed (Sheeba et al., 2008; Shang et al., 2008). Various crosses of transgenic flies demonstrated that the enhancement of nocturnal activities was due to hyper-excitement in l-LN$_v$s, and it occurred even without s-LNvs, which is necessary for circadian oscillation in DD (Sheeba et al., 2008; Shang et al., 2008). l-LN$_v$s are suggested to modulate arousal and sleep in clock-independent manner, and excitement of l-LN$_v$ might be inhibited during the dark period in *D. melanogaster*. In *P. terraenovae* also l-LN$_v$s may control the activity level: these neurons are excited during the light period and inhibited during the dark period. It may be that in flies in which the activity pattern showed the masking effects after site 2 operation the surgery did not remove l-LN$_v$s but caused damage to the neural circuit inhibiting l-LN$_v$s during the dark period. Therefore, nocturnal high activities could be observed.

A plausible involvement of s-LN$_v$s in photoperiodism

Flies lacking s-LN$_v$s (site 4 operation) did not discriminate long days from short days, and diapause incidence was about 50% under both photoperiodic conditions. Because the site 3 (control) operation did not affect photoperiodism, the effects of the site 4 operation were not the result of damage to any brain tissue. This suggests that the tissue of the site 4 contained neurons playing some roles in photoperiodism.

One may surmise that absence of neurons important for photoperiodic induction of diapause will result in entire non-diapause or diapause phenotypes. Actually, removal of pars lateralis neurons causes non-diapause phenotypes even under diapause-inducing conditions in some insects, including *P. terraenovae* (e.g. Shiga and Numata, 2000; Shimokawa et al., 2008) (for a review, see Shiga and Numata, 2007). The pars lateralis neurons innervating the corpus cardiacum or corpus allatum have been considered to inhibit hormonal events necessary for reproduction or development

under diapause-inducing conditions. Among the three components of photoperiodism (the photoreceptor, the photoperiodic clock, and the effecter), the pars lateralis neurons might be located in the effecter control centre. However, removal of a region containing s-LN$_v$s in *P. terraenovae* resulted in almost equal occurrence of diapause and non-diapause phenotypes. When there are no output signals from the photoperiodic clock to the effecter, because of a lack of photoperiodic clock components, the diapause phenotype could occur at random and its incidence would be expected to be about 50%. This was observed in our results and suggests that the region containing s-LN$_v$s is involved in the photoperiodic clock.

Site 4 contained s-LN$_v$s but no correlations were found between diapause incidence and the number of s-LN$_v$s removed. This observation suggests two possible mechanisms: (1) neurons other than s-LN$_v$s in site 4 are important for the photoperiodic mechanism or (2) s-LN$_v$s neurons are involved in the photoperiodic mechanism. There was a correlation between circadian rhythmicity and the number of s-LNv (Fig. 8B). When many of the s-LN$_v$s remained, circadian rhythmicity remained but photoperiodism disappeared, irrespective of the number of remaining s-LN$_v$s. This would support the first mechanism. Neural architecture, however, might be different between circadian oscillation and photoperiodism. The photoperiodic mechanism may require complex neuronal networks between s-LN$_v$s or between s-LN$_v$s and other neurons in the proximity. Therefore, even with substantial numbers of s-LN$_v$s left, ablation of the region proximal to the s-LN$_v$s might cause disturbance of the photoperiodic mechanism. In this scenario, the second mechanism is also probable. In a previous study synaptic connections from s-LNvs to the pars lateralis neurons, which is important for photoperiodic diapause, were demonstrated in *P. terraenovae* (Hamanaka et al., 2005). Thus, we consider that the s-LN$_v$s might send circadian timing information to the pars lateralis neurons to control the diapause phenotype. Although the current study cannot determine which possibility is more likely, it presents, for the first time, some evidence that circadian clock neurons are involved in photoperiodism. Microsurgery experiments are less accurate than molecular level techniques, however, such surgery in combination with fine-scale neuroanatomy might resolve the neural networks involved in the photoperiodic mechanism. The current results together with previous studies by Shiga and Numata (Shiga and Numata, 2000) and Hamanaka et al. (Hamanaka et al., 2005) suggest that a neural connection between s-LN$_v$s and pars lateralis neurons may be involved in photoperiodic mechanisms in *P. terraenovae*.

In *D. melanogaster*, although critical day length was shifted to a shorter value than in wild-type flies, adult females of the *per^0* strain showed photoperiodic control of reproductive diapause (Saunders et al., 1989). Saunders et al. (Saunders et al., 1989) mentioned that the *per* locus is not causally involved in the time measurement and that the crucial genes lie in different loci of the genome. In *C. costata*, it has been shown that another clock gene *timeless* is crucial for photoperiodic control of larval diapause. In a non-photoperiodic-diapause (NPD) strain of *C. costata*, of which a single autosomal gene locus encoding *tim* was mutated, both circadian eclosion rhythms and photoperiodic control of larval diapause were lost (Pavelka et al., 2003). Analysis of *tim* mRNA and TIM protein in the larval brain indicated that regulated transcription of *tim* in two brain neurons was required for photoperiodic induction of diapause in *C. costata* (Stehlik et al., 2008). Studies on *D. melanogaster* have suggested that *tim* directly affects the incidence of diapause through circadian photoreception (Tauber et al., 2007; Sandrelli et al., 2007). These studies show involvement of circadian clock genes *per* and *tim* in photoperiodic

control of diapause. The present study examined involvement of circadian clock neurons, and raised a possibility that circadian clock neurons, s-LN$_v$s, active in behavioural rhythms are also involved in photoperiodism. Our data support an idea that circadian behavioural rhythms and photoperiodism share neural elements in their underlying mechanisms. An examination of molecular level events in LN$_v$s by comparing the expression patterns of *per* and *tim* would be an interesting next step in resolving the relationships between circadian rhythms and photoperiodism.

LIST OF ABBREVIATIONS

DD	constant darkness
DN$_l$	lateral dorsal neuron
DN$_m$	medial dorsal neuron
l-LN$_v$	large ventral lateral neuron
L:D	light: dark
LN$_d$	dorsal lateral neuron
NPD	non-photoperiodic-diapause
PDF	pigment-dispersing factor
s-LN$_v$	small ventral lateral neuron

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