The Journal of Experimental Biology 204, 3303–3310 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
JEB3508

Localization of the clock controlling circadian rhythms in the first neuropile of the optic lobe in the housefly

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Accepted 2 July 2001

Summary

The visual system of a fly expresses several circadian rhythms that have been detected in the photoreceptors of the compound eye and in the first neuropile, the lamina, of the underlying optic lobe. In the lamina, axons of two classes of interneuron, L1 and L2, exhibit cyclical size changes, swelling by day and shrinking by night. These rhythmic size changes may be generated by circadian oscillators located inside and/or outside the optic lobe. To localize such oscillators, we have examined changes in the axonal cross-sectional areas of L1 and L2 within the lamina of the housefly (*Musca domestica*) under conditions of 12 h of light and 12 h of darkness (LD12:12), constant darkness (DD) or continuous light (LL) 24 h after the medulla was severed from the rest of the brain. After the lesion, the axon size changes of L1 and L2 were maintained only in LD conditions, but were weaker than in control flies. In DD and LL conditions, they were eliminated. This indicates that circadian rhythms in the lamina of a fly are generated central to the lamina and medulla neuropiles of the optic lobe. Cyclical changes of light and darkness in LD conditions are still able, however, to induce a weak daily rhythm in the axon sizes of L1 and L2.

Key words: visual system, lamina, plasticity, *Musca domestica*, *Drosophila melanogaster*.

Introduction

Circadian rhythms exist in most biological processes of living organisms, maintaining their timing and synchronising them to the diurnal changes of day and night that occur in the external environment. These rhythms have been studied in relation to biochemical and physiological processes and also in relation to the behaviour of animals, particularly in relation to their locomotor activity (Saunders, 1982). Circadian rhythms are specific daily oscillations that are temperature-compensated but, when maintained under constant conditions, have a period longer or shorter than 24 h (approximately a day). Under day/night conditions, they are entrained to the external changes of the day and night and their period then becomes equal to 24 h. Circadian rhythms are controlled by a group of genes, the so-called 'clock genes', the expression of which is cyclical. These genes are responsible for the generation of endogenous circadian oscillations by controlling circadian expression of other 'clock-controlled genes' (Hall, 1995; Young, 1998).

Circadian rhythms in behaviour are generated in cells termed 'clock neurons'. The presence and location of these neurons have been identified in many species by testing the persistence of circadian rhythms in locomotor activity after lesions of different brain structures (Stephan and Zucker, 1972), and by recording spontaneous circadian oscillations in neuronal electrical activity (Groos and Hendriks, 1982; Green and Gillette, 1982; Welsh et al., 1995). Clock neurons have been found in the suprachiasmatic nuclei of the hypothalamus in mammals (Klein et al., 1991), in insect brain (Tomioka and Chiba, 1992) and in the eyes of molluscs (Jacklet, 1989; Block et al., 1993), and these structures are termed circadian pacemakers. In addition, it has been found in both the fruit fly *Drosophila melanogaster* and in mammals that clock neurons express clock genes (Siwicki et al., 1988; Sun et al., 1997; Tei et al., 1997). In these cases, however, the products of the clock genes have been detected not only in neurons but also in glial (Siwicki et al., 1988) and other non-neuronal (Hege et al., 1997) cells, indicating that the circadian system is highly complex, comprising many circadian oscillators (Plautz et al, 1997; Yamazaki et al., 2000).

In flies, the circadian clock that regulates the temporal aspects of behaviour has best been described in *Drosophila melanogaster* (Hall, 1995; Helfrich-Förster, 1996; Young, 1998). It is composed of two groups of neurons, the dorsal and ventral lateral neurons (LNs) located in the protocerebrum (Siwicki et al., 1988; Zerr et al., 1990; Ewer et al., 1992). They all express clock proteins and some also express a neuropeptide pigment-dispersing factor (PDF), a β-homologue of pigment-dispersing hormone in crustaceans (Rao and Riehm, 1989). On the basis of this co-localisation and other evidence, it has been suggested that PDF is a transmitter of circadian information in

the output pathways from the clock to the target neurons in the visual system and elsewhere (Helfrich-Förster, 1995; Pyza and Meinertzhagen, 1997b; Renn et al., 1999). In the housefly *Musca domestica*, the circadian clock that regulates locomotory activity is known to be located outside the optic lobe, since lesion of the optic lobes does not disturb the circadian rhythm in locomotor activity (Helfrich et al., 1985). The exact location of the clock in the housefly and the expression of clock genes in the brain are, however, not known, and the *period* (*per*) gene in the housefly, one of the clock genes, has only recently been cloned (Piccin et al., 2000). In the brain of the housefly, however, cells and processes immunoreactive to PDF have been reported (Pyza and Meinertzhagen, 1997b). Two groups of cells, one group with small somata and the second with large somata, have been detected in the proximal medulla (Pyza and Meinertzhagen, 1997b). Their location is similar to that of PDF-containing cells that also contain PER protein in *Drosophila melanogaster* (Helfrich-Förster, 1995), suggesting that there may be other similarities between the two fly species.

Although circadian rhythms, other than in behavioural phenotypes, have not been intensively studied in insects, circadian oscillations exist in many systems, including the visual system. In the insect compound eye, circadian oscillations have been found in the structure of the retinal photoreceptors (Blest, 1988) and in the amplitude of the electroretinogram (ERG) (Wills et al., 1985; Chen et al., 1999). The retina of the housefly does not show clear circadian oscillations in the ERG (E. Pyza and S. R. Shaw, unpublished data), and such changes emerge only slowly over some days in the blowfly *Calliphora vicina* (Chen et al., 1999). The structure of the rhabdomeres in another muscoid fly, *Lucilia cuprina* (Williams, 1982), also fails to show obvious changes during the day and night. Circadian rhythms have, however, been found beneath the compound eye of the fly, in the first optic neuropile, the lamina, of the optic lobe (Pyza and Meinertzhagen, 1993; Pyza and Meinertzhagen, 1995; Pyza and Meinertzhagen, 1997a).

In the lamina, two classes of monopolar cell interneuron, L1 and L2, have lamina axons that change their calibre during the day and night. These cells receive direct synaptic input from the photoreceptors of the compound eye (Strausfeld and Campos-Ortega, 1977; Nicol and Meinertzhagen, 1982) and filter, enhance and transmit visual information (Laughlin and Osorio, 1989) to the next higher-order interneurons in the visual pathway. As previously shown in three fly species, *Musca domestica* (Pyza and Meinertzhagen, 1995), *Drosophila melanogaster* (Pyza and Meinertzhagen, 1999a) and *Calliphora vicina* (Pyza and Cymborowski, 2001), L1 and L2 show circadian rhythms in changes in their size and shape. In the housefly, these cells swell during the day and shrink at night and, because these cyclical changes persist in constant darkness (DD) as well as in continuous light (LL) (Pyza and Meinertzhagen, 1995), they are endogenous in origin and regulated by the circadian system. The origin of the circadian input to the L1 and L2 cells is, however, not known. It is possible that they receive circadian input from a putative clock located in the central brain; alternatively, or possibly in addition, the rhythms may be regulated by circadian oscillators located in the lamina itself, where L1 and L2 are located, or in the second neuropile, the medulla, where they terminate. To address this question, we have studied the sizes of L1 and L2 under conditions of 12 h of light and 12 h of darkness (LD), constant darkness (DD) and constant light (LL), after lesions to the optic lobe that isolate it from the rest of the brain.

Materials and methods

1-week-old female houseflies (*Musca domestica* L.) were used in this study. They originated from a laboratory culture reared under a L:D regime of 12 h light:12 h dark (LD), at a constant temperature of 25±1 °C and at constant humidity. The beginning of the day (Zeitgeber time 0, ZT0) was 09:00 h and night (ZT12) started at 21:00 h. Flies were fed on a diet containing sugar, milk powder and water.

Before surgery, flies were entrained for 2 days in LD conditions and then held for 5 days in LD conditions, constant darkness (DD) or continuous light (LL). After 5 days, the optic lobe of one side of the brain in these experimental flies was severed at the second, or internal, chiasma between the medulla and more central neuropiles of the lobula complex (Fig. 1A). The surgery was conducted in a frontal plane from the back of the head using fine microsurgery scissors (Mini-Vannas; Fine Science Tools Inc.). The lesion was judged to be complete if the parts of the optic lobe distal to the lesion separated from the proximal parts, which were still connected to the brain. In control animals, held under the same light regimes as the experimental groups, only the head capsule was opened. After surgery, the head capsule was sealed with Parafilm. Lesions were carried out at ZT1 (1 h after the beginning of daytime) or ZT13 (1 h after the beginning of night time) in LD conditions and at corresponding times, CT1 (circadian time 1, 1 h after the beginning of subjective day) and CT13 (1 h after the beginning of subjective night), in DD or LL conditions. At ZT13 and in DD, both of which are in the dark, this surgery was performed under deep red light, to which the flies were apparently insensitive (Pyza and Meinertzhagen, 1996). Before surgery, flies were immobilized by cooling on ice; they were then fixed with a piece of dental wax to a small platform for the surgery procedure itself. 24 hours after surgery, those flies that had survived were fixed for light microscopy using previously reported fixation methods (Pyza and Meinertzhagen, 1995). The heads were first fixed in a paraformaldehyde–glutaraldehyde fixative followed by osmication and embedding in Poly/Bed 812. Tangential 1 μm sections of the lamina were cut, and those in the proximal region of the lamina (Fig. 1B) were collected. They were examined with a 100×/1.25 Planapochromat oil-immersion lens, and selected images were captured in digital format using a Cohu Rs-170/CCIR monochrome CCD camera. The cross-sectional areas of the axon profiles of L1 and L2 in the proximal region of the lamina were measured using computer-based morphometric software (NIH Image), selecting the profiles of L1 and L2 (Fig. 1C) from the three rows of cartridge cross sections from

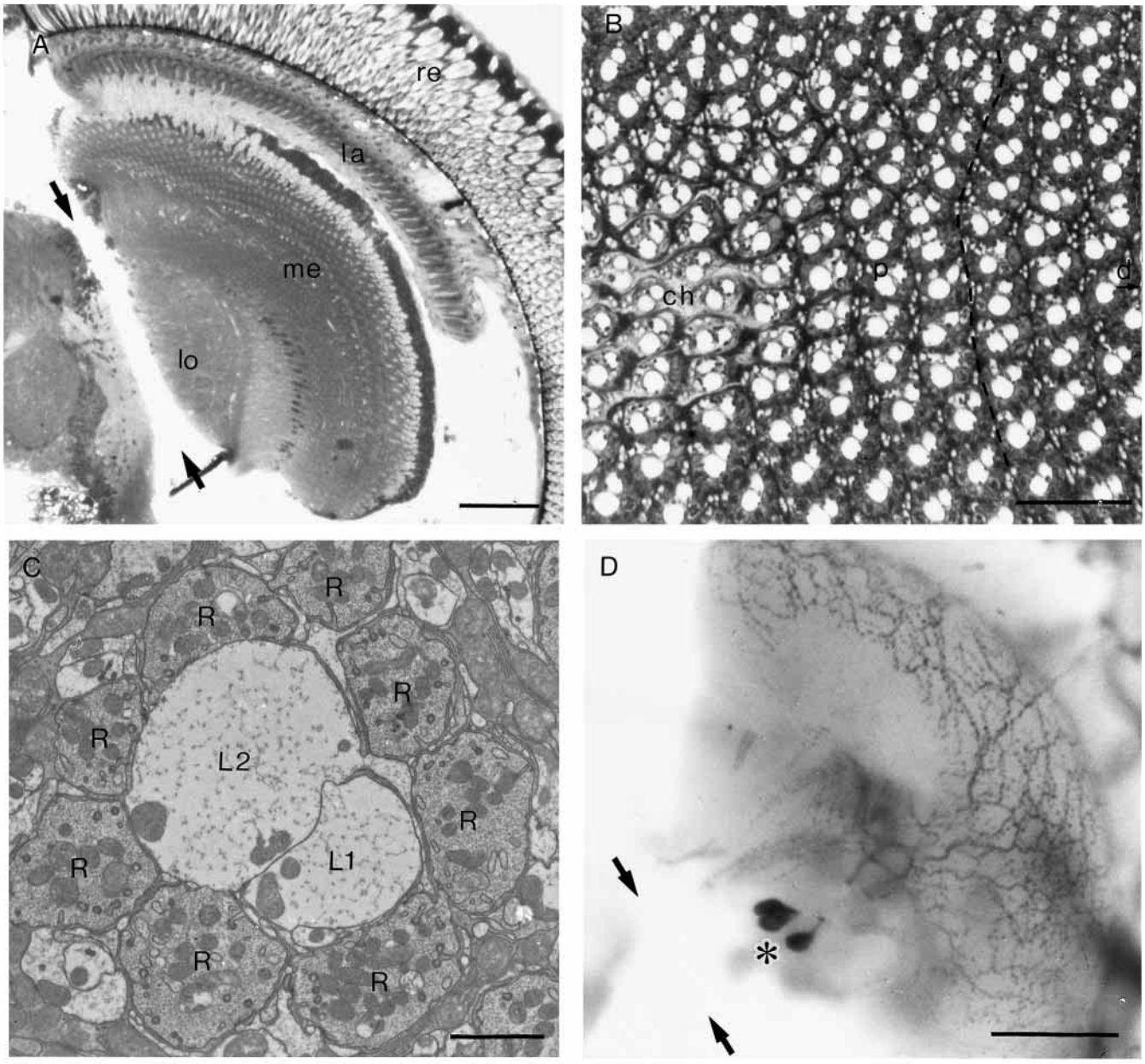

Fig. 1. (A) Frontal section of the optic lobe of *Musca domestica*. re, retina; la, lamina; me, medulla; lo, lobula. Arrows indicate the site of lesioning. Scale bar, 100 µm. (B) Light micrographs of the proximal lamina at the level of the external chiasma (ch), showing cross- sectioned photoreceptor terminals at proximal (p) and distal (d) depths in the lamina neuropile. Scale bar, 20 µm. (C) A single cartridge of the lamina comprises photoreceptors (R) and five monopolar cells. Two large monopolar cells, L1 and L2, are located at the axis of every cartridge. Scale bar, 1 µm. (D) The optic lobe of the housefly after lesioning stained with anti-PDF serum. The large PDF cell bodies (asterisk) and processes in the medulla are present in a cut-off part of the optic lobe. Arrows indicate the site of lesioning. Scale bar, 100 µm.

both the dorsal and ventral sides that surround the perimeter of the grazing tangential section of the external chiasma (Fig. 1B). Only cells that showed no sign of degeneration were examined. The methods were as previously reported (Pyza and Meinertzhagen, 1995; Pyza and Meinertzhagen, 1996).

Immunocytochemistry

To examine whether PDF-immunoreactivity still persisted in cells and processes in the part of the optic lobe that had been separated for 24 h, immunostaining was performed using anti-PDH serum (Fig. 1D). Control and experimental brains (N=7 in each group) were first incubated with primary antibody (a gift from Dr R. Rao, University of West Florida, Pensacola, USA), followed by incubation with the secondary goat anti-rabbit antibody conjugated with Cy3 fluorochrome (Jackson Immunoresearch) according to methods published previously (Pyza and Meinertzhagen, 1997b).

Statistical analysis

In each experimental or control group held in LD, DD or LL

conditions, 13–16 flies were examined, and L1 and L2 cell axons were measured from 30–40 cartridges in each fly. Next, the mean cross-sectional area of the axon profiles of L1 and L2 in the proximal lamina was calculated for each fly. The statistical significance of differences between groups was examined as a mean of means using analysis of variance (ANOVA) followed by the Scheffé test with the level of significance set at $P<0.05$. Values in the text are presented as means ± S.E.M. (standard error of mean).

Results

The validity of these experiments rested on the accuracy of the lesion that parted the compound eye and its two most distal neuropiles, the lamina and medulla, from the rest of the brain. This was first ascertained by examining the brains of lesioned flies from horizontal and frontal sections using Poly/Bed 812-embedded preparations. The results of typical lesions are shown in Fig. 1A. The plane of section cut the second chiasma between the neuropiles of the medulla and lobula complex. Because of the curvature of the medulla, there was some damage to this neuropile, but only in its distal part at the dorsal and ventral margins, and a small part of the lobula complex was left distal to the lesion (Fig. 1A).

In sham-operated flies held in LD conditions, the day/night differences measured at ZT1 and ZT13 were 22 % for L1 and 45 % for L2 (Fig. 2A,B). After the optic lobe had been severed from the rest of the brain, the axon cross-sectional area of L1 in the lamina was larger by 8 % and that of L2 by 25 % in flies that suffered the lesion at ZT1 (L1, 5.87±0.47 μm^2; L2, 12.94±1.29 μm^2) than in flies lesioned at ZT13 (L1, 5.40±0.87 μm^2; L2, 9.72±1.25 μm^2). Both cells were larger during the day than during the night in both experimental and control flies, although these differences were statistically significant only for L2 in control flies ($P<0.0402$). The axons of L1 cells in sham-operated flies were smaller by 13 % than those of experimental flies after a lesion at ZT1, while the size of L2 axons was unchanged. Larger differences in the sizes of L1 and L2 axons between lesioned and sham-operated flies were observed after a lesion at ZT13, when the cross-sectional areas of L1 and L2 axons were both smaller in sham-operated flies, L1 by 33 % and L2 by 38 %. This means that the reduction in amplitude of axon size changes occurring after surgery mainly results from the fact that the axons of L1 and especially L2 do not shrink during the night as effectively as cells in control animals.

In sham-operated flies in DD conditions, the cross-sectional area of L1 cell axons was larger by 20 % at CT1 than at CT13 while the increase for L2 cells was 53 %. These differences were statistically significant ($P<0.001$) (Fig. 2A,B). In flies examined in constant darkness in our previous study (Pyza and Meinertzhagen, 1995), L1 and L2 were also larger during the subjective day than during the subjective night but these differences were statistically significant only for L2.

In experimental flies, held in DD conditions, the axon cross-

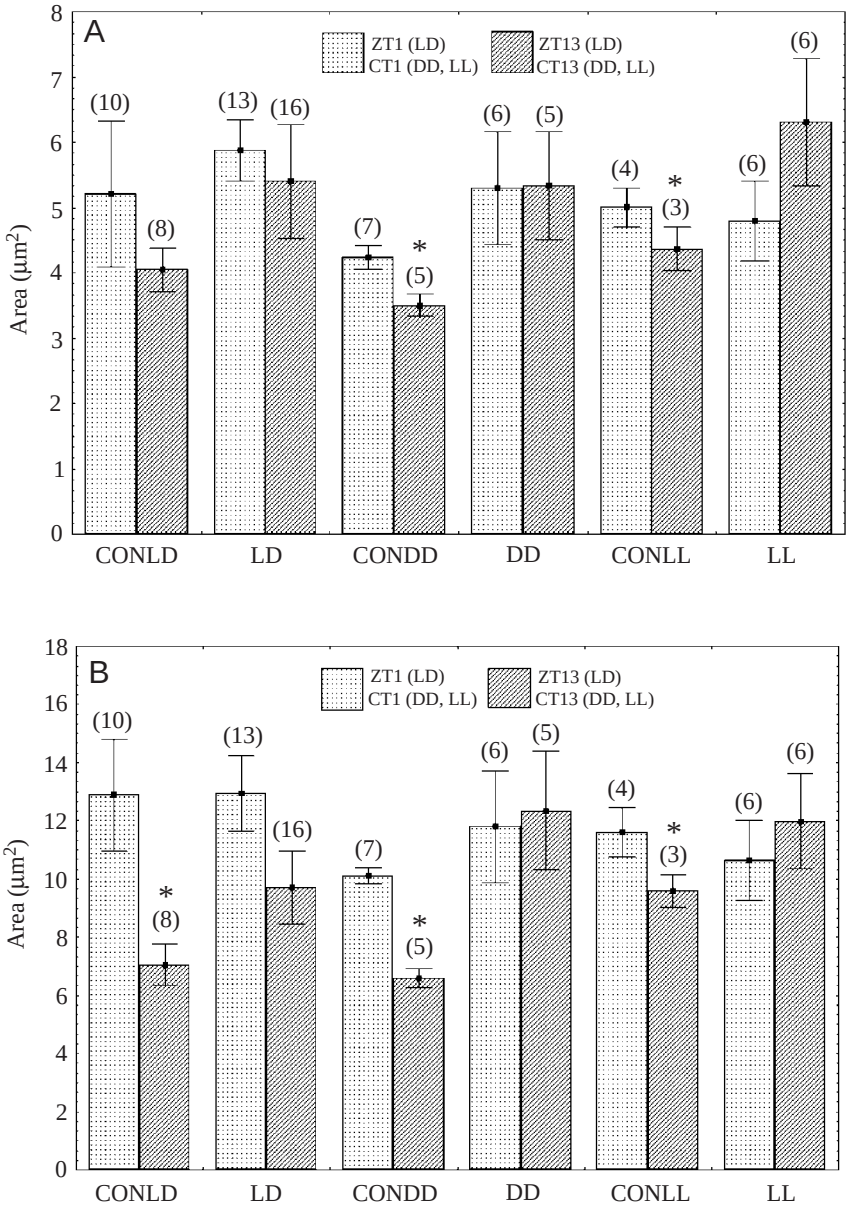

Fig. 2. The axonal cross-sectional areas of L1 (A) and L2 (B) monopolar cells in control (CONLD, CONDD, CONLL) and experimental (LD, DD, LL) flies under light and dark conditions (L:D 12 h:12 h), measured at ZT1 and ZT13 and in flies maintained in constant darkness (DD) and continuous light (LL) measured at CT1 and CT13. Values are means ± S.E.M. The number of flies in each group is given at the top of each column. The asterisks indicate statistically significant differences.

sectional areas of L1 cell axons in flies in which the lamina and medulla were separated at CT1 from the central brain was the same as in flies lesioned at CT13 (Fig. 2A). In the case of L2, the cross-sectional areas of the axons were slightly smaller after lesion at CT1 than after the same lesion, at CT13, but only by 5 % (Fig. 2B). After lesions in constant darkness, therefore, both cells had similar sizes during the subjective day and subjective night because they were unable to shrink at CT13. Thus, the lesioned flies in the present study had lost the circadian rhythm of their size changes seen in sham-operated and in intact flies. Even though the mean cross-sectional area of the axons of L1 and L2 in experimental flies in DD had similar sizes at CT1, they were larger at CT13 when compared with their areas at both ZT1 and ZT13 in LD conditions in both experimental and control groups. Both cells were also larger at CT1 and CT13 in lesioned than in sham-operated flies held in DD conditions.

In sham-operated flies in LL conditions and in flies examined under these conditions in our previous study (Pyza and Meinertzhagen, 1995), the cross-sectional areas of the axons of L1 and L2 were greater during the subjective day than during the subjective night. In sham-operated flies, axons of L1 and L2 were larger during the subjective day than during the subjective night by 14.4 % in the case of L1 and by 21.17 % for L2. These differences were statistically significant (P<0.001). In our previous study under LL conditions, a statistically significant difference was detected only for L2 (Pyza and Meinertzhagen, 1995).

In experimental flies, held in constant light, LL, the mean axon size of L1 tended to be smaller by 32 % in flies lesioned at CT1 than in flies in which the lamina and medulla were parted from the central brain at CT13 (Fig. 2A), while the axon of L2 was 13 % smaller after the CT1 lesion than the CT13 lesion (Fig. 2B). Neither of these differences was statistically significant, however. The pattern of size changes of L1 and L2 observed in LD conditions was moreover reversed after lesions under LL conditions. In the lesioned flies from this study, the cross-sectional areas of L1 and L2 axons were larger than in control flies at CT13 but smaller at CT1. This means that, after lesion under constant light conditions, cells not only do not shrink at CT13 but actually increase in size. Since our previous study (Pyza and Meinertzhagen, 1995) showed that continuous light increases the sizes of L1 and L2, the observed increase in their size at CT13 after surgery in the present study could result from both the effect of LL conditions on cells and the lack of the circadian mechanism that normally shrink cells during the subjective night.

Immunocytochemical studies carried out on experimental brains with PDH antibody showed some large PDF-reactive cells and processes in the medulla separated from the rest of optic lobe (Fig. 1D). During lesioning, the connecting pathways between two optic lobes through the posterior optic tract (POT) were cut and small PDF-reactive medulla neurons failed to survive, since they cannot be detected in any animals following lesion. In the brain of the housefly, small PDF-reactive cells are located ventrally at the base of the medulla, very close to the protocerebrum. These neurons connect with a thick bundle of fibres that also has connections with the somata of the large PDF-reactive cells. The fibres from this bundle extend to the medulla and the lamina and to the dorsal protocerebrum, as well as to the posterior optic tract. Since the lesion was carried out at the level of the second chiasma, the neurites of the small PDF-reactive neurons fell in the plane of the lesion. This means that the clock and/or its output pathways were destroyed, since these cells are possible candidates for the clock neurons in the housefly (E. Pyza, unpublished data).

Discussion

The results obtained in the present study show that a lesion of the optic lobe that separates the retina and the outer two optic neuropiles, the lamina and medulla, from the rest of the brain abolishes circadian rhythms in the size changes of L1 and L2 axons in the lamina. Since sham-operated flies under DD and LL conditions maintain these rhythms, the lesion must separate the cells from a more centrally located oscillator that controls them. Only in the normal light cycle, LD, are daily rhythms still maintained after such lesions, so that cells remain larger during the day and smaller during the night, resembling those changes seen in intact flies (Pyza and Meinertzhagen, 1995). Under LD conditions, however, such changes have a smaller amplitude than in sham-operated flies, while under conditions of constant darkness they are completely abolished, so that the axons are equal in size during the subjective day and the subjective night. When the animals are exposed to constant light, LL, the cross-sectional area of the axons of L1 and L2 are larger during the subjective night than the subjective day so that the pattern of axon size changes is actually reversed compared with the pattern in flies under LD conditions.

The most obvious interpretation for the lack of any significant changes in the axonal cross-sectional area of L1 and L2 during the subjective day and the subjective night under DD and LL in lesioned flies, is that the rhythm is disrupted because circadian information no longer reaches L1 and L2. Alternatively, it could be that the rhythm persists, but that in some way the lesion changes its period and phase so that the rhythm of L1 and L2 size changes is no longer visible. Although theoretically it would be possible to measure the period and phase of the rhythm in L1 and L2, there are several reasons why this would not be practicable. First, it would require examination at least four times a day for 2 days prior to the lesion and 2 days after the lesion, instead of the two time points examined here. In addition, several flies would have to be examined for each time point, because each fly could be in a different phase of its rhythm, or might have a different period to its rhythm, after the lesion. We therefore could not examine the question of changes in the period or phase of the rhythm of axon size changes in L1 and L2, so this alternative remains a formal possibility.

The rhythms we see in the morphology of L1 and L2 are strongest under LD conditions and less robust in constant

darkness. We interpret this difference as a result of the fact that under LD conditions these cells receive two inputs: exogenous, the pattern of light and dark, and endogenous, the circadian input from the clock entrained to LD conditions. The different sizes of the axons of L1 and L2 during the day and night probably correlate with cell activity, which seems to be higher during the day than during the night since a [^{3}H]deoxyglucose incorporation study has showed higher [^{3}H]deoxyglucose uptake in L1 and L2 during the day than during the night (Bausenwein, 1994).

In evaluating the results from the optic lobe lesions, it is important to acknowledge effects that are not of circadian origin. In the lesioned part of the optic lobe, some neurons show signs of degeneration such as shrinking and darkening of the cytoplasm. The time scale of such changes (24 h) corresponds with degeneration times seen in other insect neurons (Schürmann, 1980; Brandstätter et al., 1991) exhibiting similar ultrastructural changes. Such neurons in our case are presumably those that are the immediate synaptic partners, in either the orthograde (Brandstätter et al., 1991) or retrograde (Brandstätter et al., 1992) direction, of those neurons whose axons are lesioned in the second chiasma. We have no further evidence of their identity, however. In addition to these changes, other cells do not shrink after lesioning and, instead, are larger than in sham-operated flies, especially after lesions during the night. In principle, therefore, there could be two effects on L1 and L2 after the lesion: cell degeneration, which causes shrinking, and a circadian effect during the day, which causes cell enlargement, possibly resulting in the large deviations from the means of axon size that were observed. The large deviations in axon cross-sectional areas for L1 and L2 could be the result of individual differences introduced by surgery itself and desynchronization of the rhythms in L1 and L2 between different cartridges as a result of lack circadian input from the brain and/or the contralateral optic lobe. As in other insect species, for example the cricket *Gryllus bimaculatus* (Tomioka et al., 1994), the circadian clock of *Musca domestica* may be composed of two oscillators that can exchange circadian and light information.

The site of the lesion was selected so that the clock that controls the rhythms in the optic lobe of *Musca domestica* could be localized. We severed the optic lobe at the level of the second chiasma to avoid damaging the axons of L1 and L2 and, since L1 and L2 have somata in the lamina cortex and terminals in the distal medulla, to leave these cells intact in the distal part of the optic lobe. This lesion also leaves intact the contralateral optic lobe and the protocerebrum.

A study in which both optic lobes were lesioned was carried out in an attempt to localise the clock that generates the circadian rhythm in locomotor activity observed in the fly (Helfrich et al., 1985). In that study, lesions of the optic lobes were carried out near the protocerebrum, and the treated flies still showed a clear circadian rhythm in behaviour. The study by Helfrich et al. (Helfrich et al., 1985) confirms that the clock for circadian rhythms in locomotor activity must be located in the central part of the brain and not in the optic lobes, as is the case in cockroaches (Nishiitsutsuji-Uwo and Pittendrigh, 1968; Roberts, 1974; Sokolove, 1975; Page, 1984; Stengl and Homberg, 1994) and crickets (Tomioka and Chiba, 1986; Tomioka and Chiba, 1992). In the case of cockroaches and crickets, however, the clock is not located in the same site; in the cricket *Gryllus bimaculatus* it has been localised in the lamina–medulla complex (Tomioka and Chiba, 1986; Tomioka and Chiba, 1992), whereas in the cockroach *Leucophaea maderae* it is localised in the accessory medulla, in the proximal part of the medulla (Reischig and Stengl, 1996).

Although some of these differences may be attributed to the different geometries of optic neuropiles, it is clear that one cannot extrapolate between species in this matter. Our results provide evidence that circadian rhythms in the lamina of flies are generated outside the optic lobe, or at least outside the retina and the lamina and medulla neuropiles, and that the location of the clock may be similar to that of the clock driving locomotor activity, even though it is known that both rhythms are driven by different oscillators (Pyza and Cymborowski, 2001). Since the large PDF cells are left distal to the lesion and are not separated from the lamina cells, and since the circadian rhythms in the morphology of L1 and L2 are nevertheless lost, then their rhythms cannot normally come from the large PDF cells, at least not from these cells located in the ipsilateral optic lobe. Instead, they may result from coupling, disrupted by the lesion, between the PDF cells of both optic lobes. Such a coupling between oscillators located in two optic lobes is essential for the circadian rhythm of locomotor activity in cockroaches (Page, 1983) and is also important for the overt circadian rhythms in other species (Ushirogawa et al., 1997).

If circadian input is stopped, as when we lesion the optic lobe at the level of the internal chiasma, the axons of L1 and L2 still change their sizes, since cyclical changes in light and darkness during the day and night continue to induce a weak exogenous rhythm of size changes.

The location of the clock neurons in *Musca domestica* appears to be similar to that of equivalent cells in *Drosophila melanogaster*, but the methods used to identify this location in each species differ considerably. In *Drosophila melanogaster*, the essential role played by the lateral neurons (LNs) was demonstrated by correlating the expression of behavioural rhythmicity with the pattern of *per* expression in mosaic flies (Ewer et al., 1992). While this method involves no surgical interference in the brain and allows a high level of anatomical precision, the expression of *per* is chronic. In our experiments, surgical lesions have been used that are, by comparison, anatomically less precise, but that deny access of the lamina to candidate clock neurons, thus allowing observation of effects that are acute. Neither approach is reversible, unlike the situation in cockroaches where clock neurons can regenerate to restore circadian rhythmicity (Stengl and Homberg, 1994). Closer localisation of the circadian clock in the housefly using the approaches adopted in this report will have to await further refinement of cell ablation methods, preferably coupled to a more rapid method than cell morphometry of assaying the circadian state of the lamina.

Details of the molecular mechanism for the circadian clock of the housefly may differ significantly from those for *Drosophila melanogaster* (C. P. Kyriacou, personal communication). In *Drosophila melanogaster*, the clock neurons, LNs, are located in the lateral protocerebrum and are necessary to express a circadian rhythm of locomotory activity (Ewer at al., 1992; Frisch et al., 1994; Helfrich-Förster, 1998). There are, however, indications that other neurons expressing *per* also participate in regulating behavioural rhythms. For example, neurons in the optic lobes expressing *per* may stabilise these rhythms at that site (Helfrich, 1986). Examination of, and comparisons between, *Drosophila melanogaster* mutants expressing *per* either exclusively in the LNs, or in the LNs and the compound eye photoreceptors, show that the rhythms of axonal size changes in L1 and L2 depend on *per* expression not only in the brain, but also in the retina and probably also in glial cells in the lamina (Pyza and Meinertzhagen, 1999b). For behavioural rhythms, however, LNs are essential and some of them colocalise with PDF, which has recently been suggested as a transmitter of circadian information to target cells not only in *Drosophila melanogaster* (Helfrich-Förster, 1995; Helfrich-Förster, 2000) and *Musca domestica* (Meinertzhagen and Pyza, 1996) but also in cockroches (Stengl and Homberg, 1994; Petri and Stengl, 1999). Pigment dispersing factor also has an effect on L1 and L2 in the lamina and injection of this peptide increases the sizes of both cells, mimicking their swelling during the day (Pyza and Meinertzhagen, 1996). Moreover, the immunoreactive varicosities of PDF fibres in the lamina and medulla exhibit size fluctuations under LD and DD conditions that are compatible with peptide release (Pyza and Meinertzhagen, 1997b). If some or all of the PDF-containing cells in *Musca domestica* are also clock neurons, it is possible that such a role may be played by the small PDF cells since these cells were not present in the lesioned part of the optic lobe in the present study, whereas the large PDF cells were still present in the distal part of the optic lobe. It is also possible that absence of interactions between the large PDF cells located in ipsilateral and contralateral optic lobes after lesion results in disabled circadian input to the lamina.

This work was supported by the International Programme of the Howard Hughes Medical Institute (HHMI 75195-543102) and the Jagiellonian University (DS/IZ/MUZ/2000).

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