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Behavioral and molecular analyses suggest that circadian output is disrupted by *disconnected* mutants in *D.melanogaster*

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Mutations in the *disconnected* (*disco*) gene act to disrupt neural cell patterning in the *Drosophila* visual system. These mutations also affect adult locomotor activity rhythms, as *disco* flies are arrhythmic under conditions of constant darkness (DD). To determine the state of the circadian pacemaker in *disco* mutants, we constructed with per^S double mutants (a short period allele of the period gene) and assayed their behavioral rhythms in light-dark cycles (LD), and their biochemical rhythms of *period* gene expression under both LD and DD conditions. The results demonstrate that *disco* flies are rhythmic, indicating that they have an active circadian pacemaker that can be entrained by light. They also suggest that *disco* mutants block or interfere with elements of the circadian system located between the central pacemaker and its outputs that mediate overt rhythms.
Key words: behavior/circadian rhythms/*Drosophila*/RNA cycling

Introduction

Circadian rhythms are generally described as having three components: an entrainment or input pathway linking the environment to the circadian pacemaker, the pacemaker itself, and output pathways that connect the pacemaker to various physiological and behavioral processes. To determine the biochemical mechanisms that underlie the circadian clock, it is necessary to define molecules that contribute to these different components. One means of identification is through the characterization of mutations that disrupt circadian rhythms. Such an analysis has been undertaken in *Drosophila melanogaster* where several genes that affect circadian rhythms have been identified (review: Hall and Rosbash, 1987).

The best studied of these is the *period* (*per*) gene. Various lines of evidence suggest that this gene is central to circadian pacemaker function. *per* influences both qualitative and quantitative aspects of behavioral rhythmicity. Single amino acid changes in the *per* protein can shorten (per^S) or lengthen (per^L) the free-running circadian period in constant darkness (DD), whereas loss of functional *per* protein (per^{01}) abolishes rhythmicity under these conditions (Konopka and Benzer, 1971). These effects are seen in circadian rhythms of individual adult flies (locomotor activity rhythms) and of populations (eclosion) (reviews: Hall and Rosbash, 1988; Rosbash and Hall, 1989; Young *et al.*, 1989). As entrainment of the circadian pacemaker also appears to require the *per* gene (Ewer *et al.*, 1990), our current view is that *per* expression is necessary for flies to tell or to keep time.

Circadian fluctuations in the levels of *per* mRNA and *per* protein have recently been described (Siwicki *et al.*, 1988; Hardin *et al.*, 1990; Zerr *et al.*, 1990; Zwiebel *et al.*, 1991). These oscillations persist under DD conditions and are therefore likely to be *bona fide* molecular circadian rhythms. Several observations show that mutant *per* alleles influence the phase and period of *per* RNA cycling in a manner that parallels their effects on behavioral rhythms, indicating that the *per* protein participates in a feedback loop that affects the circadian cycling of its own mRNA (Hardin *et al.*, 1990). Because this feedback loop is associated with a gene essential for circadian clock function and is operational in an anatomical region known to contain the pacemaker, i.e. the head (Handler and Konopka, 1979; Konopka *et al.*, 1983; Ewer, 1990), it may be an important component of the circadian pacemaker that affects behavioral rhythms. If not, *per* RNA cycling can at least be used as an experimental tool to reflect the running of a circadian clock.

This paper describes the effects of mutants in the *disconnected* gene (*disco*) on the behavioral and molecular manifestations of circadian clock function. Like the arrhythmic per^{01} mutant, *disco* mutations have dramatic effects on circadian rhythms; for example, under free-running conditions, mutations at this locus cause virtual arrhytmicity in both eclosion and locomoter activity (Dushay *et al.*, 1989). *disco* mutations were originally isolated based on their drastic effects on the morphology of the visual system (Steller *et al.*, 1987). Therefore, it was anticipated that their rhythm phenotypes would be caused by a problem in the light input or entrainment pathway. Surprisingly, the experiments presented in this report suggest that *disco* mutants receive photic information in a relatively normal manner. As circadian oscillator function also appears intact in these mutant strains, the data suggest that the *disco* mutants block the clock's output pathway.

Results

Locomotor activity of disco mutants in LD

Both of the *disco* mutants that were monitored behaviorally ($disco^2$ and $disco^{1656}$) and the two per^0 alleles (per^{01} and per^{04}) were essentially arrhythmic when assayed in DD (Dushay *et al.*, 1989 and $disco^2$; P.E.Hardin, unpublished observations for $disco^{1656}$; Hamblen-Coyle *et al.*, 1989 for the per^0s). The arrhythmic *per* mutants probably eliminate or severely cripple circadian pacemaker function (e.g. as discussed by Rosbash and Hall, 1989). To gain more insight into why the *disco* mutants are also arrhythmic in DD, the

locomotor activity patterns of these flies were assayed in light–dark cycles (LD) and compared with those from per^{01} flies.

Under these conditions, wild-type flies have two principal active periods: the morning peak anticipates lights-on and the evening peak anticipates lights-off (Hamblen-Coyle,M.J., Wheeler,D.A., Rutila,J.E., Rosbash,M. and Hall,J.C. 1991, submitted). In contrast, the per^{01} arrhythmic strain has only a single, very broad activity peak. Importantly, per^{01} locomoter activity does not anticipate the light–dark (or dark–light) transitions but appears to increase in the presence of light and decrease in its absence. These and other data suggest that the per^{01} locomoter activity responds to light and reflects the absence of a functioning circadian clock (Petersen *et al.*, 1988; Dushay *et al.*, 1989; Hamblen-Coyle *et al.*, 1989).

Previous analyses of *disco* flies under these conditions indicated that they also have a major broad activity peak during the lights-on portion of the 24 h cycle (Dushay *et al.*, 1989). The preferred interpretation of these results was that *disco* flies are arrhythmic, and that the differences from the per^{01} pattern were due to the inability of *disco* flies to process or to respond to light information properly, presumably because of anatomical defects in the central brain (Dushay *et al.*, 1989).

Newer methods of analyzing LD behavior make it possible to average the activity patterns of a large number of flies (Hamblen-Coyle *et al.*, 1989; Dushay *et al.*, 1990). When *w disco*^{2} *f* flies were analyzed with these procedures, they manifested a morning activity peak as well as a larger evening activity peak (Figure 1). This pattern is similar to that of wild-type, in that the morning peak is centered around lights-on and the evening peak occurs prior to lights-off, but it differs from wild-type by having a broader and somewhat precocious 'evening' activity peak (Figure 1). The similarity in the activity profiles of wild-type and *disco* flies suggests the presence of an active pacemaker.

If *disco* flies contain an active pacemaker, a shift in the phase of the evening activity peak should result from the presence of a per^S or per^L mutant allele. This expectation is based on the fact that strains that carry these two alleles have relatively normal morning activity peaks, but their evening activity peaks are shifted to earlier and later times, respectively (Hamblen-Coyle, 1991, submitted). The evening activity peak of per^S *disco*^{2} *f* flies, was indeed shifted to earlier times as compared with *disco*^{2} *f* flies, indistinguishable from the magnitude of the shift observed in a per^S–wild-type comparison (Figure 1). As in the *disco*–wild-type comparison, the evening activity peak of the per^S*dico*^{2} *f* flies was broader than that of per^S flies. The results indicate that a functional circadian pacemaker is operating under LD conditions in *disco* flies.

Cycling of per RNA in disco mutants in LD

We have previously reported that *per* RNA levels undergo circadian cycling with an amplitude of 5–10 in adult heads (Hardin *et al.*, 1990). This provides an additional assay for circadian pacemaker function in *Drosophila* that is potentially independent of behavioral rhythms. The *per* RNA times course from *w disco*^{2} *f* flies manifested circadian cycling with peak values occurring shortly after lights-off (Figure 2). The amplitude, phase and breadth of the peak was indistinguishable from what was previously observed for wild-type flies (Hardin *et al.*, 1990). The RNA timecourse from per^S *disco*^{2} *f* flies was shifted at least 2 h earlier than that from *disco* or from wild-type flies (Figure 2). This shift is also in complete agreement with results previously obtained from per^S flies (Hardin *et al.*, 1990). From both the behavioral and the biochemical characterizations, we conclude that a circadian clock is running in the *disco* mutant, at least under LD conditions.

Cycling in per RNA in disco flies in DD

In wild-type flies, *per* RNA cycling also occurs in DD conditions (Hardin *et al.*, 1990). To examine RNA cycling in *disco* under these conditions, we monitored the abundance of *per* RNA in per^+ *disco*^{2} *f* and per^S *disco*^{2} *f* adults as a

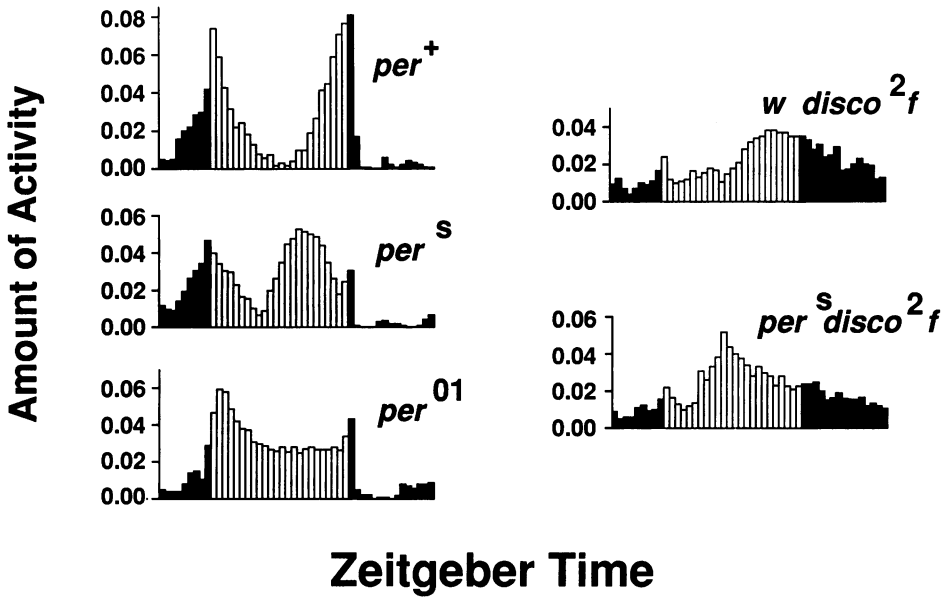

Fig. 1. LD locomotor activity. Eduction profiles are shown for per^S, wild-type, *w disco*2^2 *f*, per^S *disco*^{2} *f* and per^{01} adults. The amount of activity is measured in percent (1.0 equals 100%). The white bars represent activity during light-on and the black bars represent activity during lights-off. These eductions are averaged (i.e. activity level per 0.5 h time bin per fly) for the following number of males for each genotype: four for per per^S, five for wild-type, four for *w disco*^{2} *f*, seven for per^S *disco*^{2} *f* and four for per^{01}.

function of time (Figure 3). Both genotypes manifested *per* RNA cycling. RNA levels from per^{+} $disco^{2}$ *f* flies oscillated with a ~24 h period, whereas the fluctuations from per^{S} $disco^{2}$ *f* had a ~20 h period. For both strains, the amplitude (peak to trough values) was ~3, less than the amplitude of ~6 commonly observed for wild-type (Hardin *et al.*, 1990). The amplitude also decreased during the course of the DD run, similar to observations previously made on a wild-type strain (Hardin *et al.*, 1990). The data indicate the presence of a free-running circadian oscillator in *disco* flies, despite the absence of overt behavioral rhythms under these conditions.

Discussion

Previous behavioral testing of *disco* mutants suggested the absence of overt locomotor activity circadian rhythms. Under LD conditions, the activity patterns resembled those from arrhythmic per^{01} flies. Given the dramatic neuro-anatomical effects of this mutation, it was suggested that the arrhythmic behavioral patterns were due to difficulties in receiving or interpreting light-mediated entrainment cues (Dushay *et al.*, 1989). Yet results presented here suggest that *disco* flies contain an active, entrained circadian pacemaker that functions under both LD and DD conditions. Pacemaker function was revealed by biochemical assays under both LD and DD conditions and was also detected with behavioral assays under LD conditions.

The biochemical results are in complete agreement with previous histochemical experiments that visualized *per* protein cycling in *disco* flies both in LD and DD. Although absolute levels of *per* protein could not be determined due to the non-quantitative nature of the histochemical scoring, LD protein cycling in eye photoreceptors and in putative brain glial cells was very similar (for both phase and amplitude) from what had been previously observed in wild-type flies. Protein cycling in these two tissues was also detected in *disco* flies under DD conditions (Zerr *et al.*, 1990).

To evaluate the nature of the behavioral lesion in *disco* flies, it is useful to consider a formal model of the pathways used to control circadian activity in *Drosophila* (Figure 4). In this model light acts to affect activity directly (3) or indirectly through the entrainment pathway (1). The pacemaker is considered to communicate with activity effectors via an output pathway (4). The direct light pathway

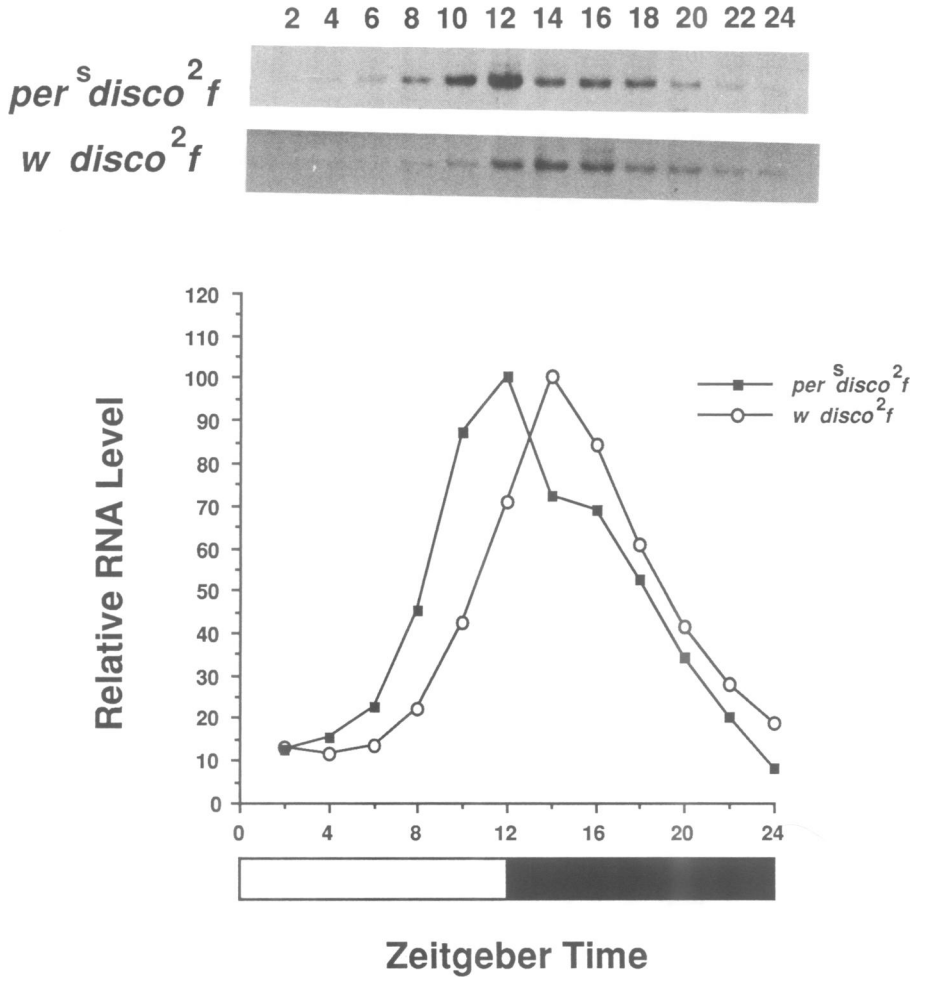

Fig. 2. LD cycling of *per* RNA in *w* $disco^{2}$ *f* and per^{S} $disco^{2}$ *f* adults. RNase protection assays were performed on *w* $disco^{2}$ *f* and per^{S} $disco^{2}$ *f* RNAs collected every 2 h during a 12 h light–12 h dark cycle. The numbers above each lane represent the number of hours after the last lights-on. The protected *per* fragment shown is a portion of exon 3. Quantitation of this data is shown graphically. Relative RNA abundance refers to the *per*/RP49 values, and the peak value was set to 100 (Hardin *et al.*, 1990). The white and black bars depict times when the lights were on versus off, respectively. The level of *w* $disco^{2}$ *f* RNA is shown as open circles; the level of per^{S} $disco^{2}$ *f* RNA is shown as solid boxes.

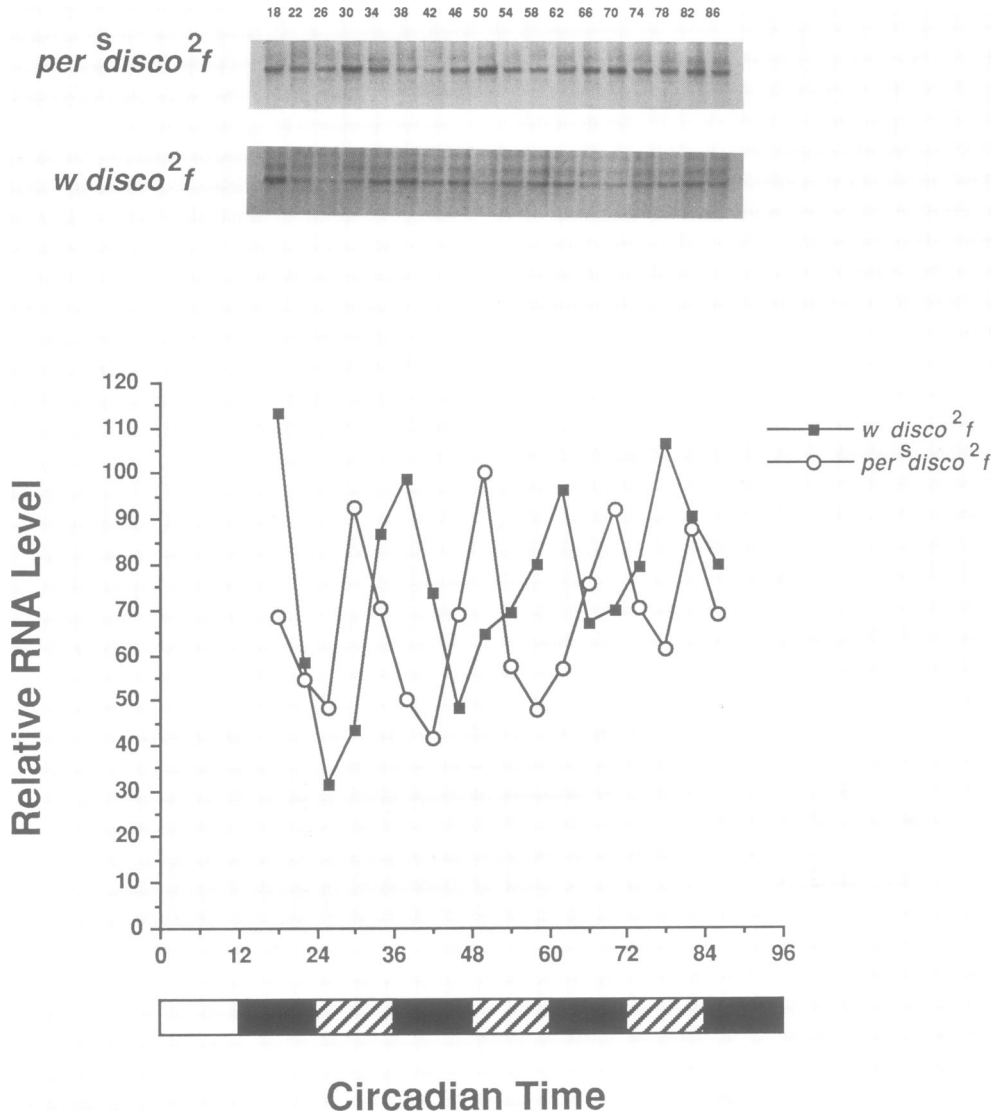

Fig. 3. DD cycling of *per* RNA in *w disco*^{2} *f* and *per*^{S} *disco*^{2} *f* flies. RNase protection assays were performed on *w disco*^{2} *f* and *per*^{S} disco^2 *f* RNAs collected every 4 h during 3 days of complete darkness. The numbers above each lane denote the number of hours after the last lights-on. The protected fragment shown is a portion of *per* exon 3. Quantitation of this data is shown in the graph below. Relative RNA abundance refers to the *per*/RP49 values, where the peak value was set to 100. The white and black bar depicts the last lights-on time period, before the flies proceeded into constant darkness (during which black bars extrapolate back to times when the lights were off in LD, and shaded bars extrapolate to lights-on time segments). The levels of *w disco*^{2} *f* RNA is shown as closed boxes; the level of *per*^{S} *disco*^{2} *f* RNA is shown as open circles.

(3) and the pacemaker output pathway (4) may have elements in common, or may even merge, upstream of their effects on the activity effectors (5). In principle, the effect of *disco* on circadian rhythms could occur at any of these five locations.

The observed circadian cycling of *per* RNA suggests that the pacemaker (2) is functional in *disco* flies. As *per* RNA (and protein) cycling may not be an intrinsic component of a putative central oscillator, this biochemical cycling may lie downstream of the central pacemaker or may even reflect a parallel circuit that is not relevant to behavioral rhythms. Nevertheless, its presence suggests that some circadian pacemaker activity is present in *disco* flies. The effects of the *per*^{S} allele on the (DD) behavioral and (LD and DD) biochemical rhythms of *disco* flies support this conclusion, although we note that the *per* product has also not been proven to be a component of a circadian pacemaker. If circadian rhythms are present, then an entrainment pathway (1) must be functional in *disco* flies. Although this entrainment pathway could be different from (and parallel to) the one used for locomotor activity rhythms, the most parsimonious conclusion from these considerations is that the basic entrainment pathway that helps mediate these activity rhythms is functional in *disco* flies.

The lack of careful controls on the light intensity used in these experiments makes it impossible to conclude that the entrainment pathway is indistinguishable from that present in a wild-type strain. However, the presence of a functional entrainment pathway in these severely eye optic-lobe-damaged mutants is reminiscent of the extensive evidence which indicates that the eyes and even the optic lobes make only minor contributions to the circadian rhythm phenotypes (Helfrich and Engelmann, 1983; Helfrich *et al.*, 1985; Helfrich, 1986; Dushay *et al.*, 1989). These experiments

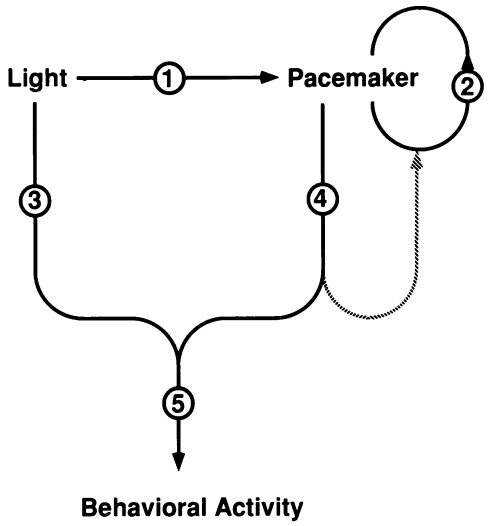

Fig. 4. The influence of light on behavioral activity. Light may affect behavioral activity either directly or through the circadian clock as shown by the solid lines. The numbers represent pathways which may be blocked by *disco* mutants. These mutants could block the circadian clock's affect on behavior within the entrainment pathway (1), the pacemaker (2) or the output pathway (4). Alternatively, *disco* mutations may block light's direct influence (3) or some elements common to the circadian clock and direct light influence (5) on behavior. The hatched line represents the effects that the output pathway may have on the circadian pacemaker.

and others are consistent with the view that an unidentified extraocular photoreceptor system is involved in light entrainment in these (and other) higher insects (reviews: Truman, 1976; Page, 1982).

To account for the fact that the behavioral, rhythms are virtually non-existent in DD despite the presence of a functional pacemaker and entrainment pathway, we suggest that it is the output pathway that is defective in *disco* flies (4 or perhaps 5). Yet the data still require an explanation for the less robust RNA cycling than is observed in wild-type under DD conditions. A possibility is that *per* RNA cycling might be part of the output pathway, and the weak cycling would then reflect the effect of *disco* on this part of the pathway (see below). If the RNA cycling is a genuine component of the central pacemaker, the weak cycling might then reflect a mildly lesioned pacemaker or entrainment pathway. Alternatively, and as previously proposed (Hardin *et al.*, 1990; Zerr *et al.*, 1990), there might be feedback effects of the output pathway on the pacemaker (i.e. these two pathways might not be entirely distinct; these would account for the effects of a mutant output pathway on apparent pacemaker activity (Figure 4). This feedback loop might manifest itself less readily under LD conditions in which the daily light fluctuations reinforce intrinsic pacemaker activity and strengthen biochemical and behavioral cycling phenomena (through pathway 1). It is interesting to consider these possibilities from a brain anatomy point of view. The effects of *disco* on behavior are recessive, in contrast to the semi-dominant nature of *per* and most other rhythm mutants (Dushay *et al.*, 1989). This difference is likely to reflect an underlying anatomical defect in *disco* instead of a physiological defect that is probably responsible for *per*'s effects (Ewer *et al.*, 1990).

In wild-type fly heads *per* protein has been shown to cycle in all three cell types where it is found, i.e. photoreceptors, putative glia, and in certain 'lateral neurons'. In *disco* heads, similar results were reported for photoreceptors and putative glia (Zerr *et al.*, 1990). For the lateral neurons, however, *per* staining was observed in only a small fraction (~15%) of *disco* individuals and could not be assayed for cycling (Zerr *et al.*, 1990). This low percentage of lateral neuron staining may be due to a lower level of *per* protein in these cells or, more likely in our view, to *disco*-mediated anatomical defects, e.g. missing or misplaced lateral neurons. *per* expression in the lateral neurons may then be part of the output pathway and required for normal or near normal locomotor activity rhythms.

Materials and methods

Drosophila strains

All fly stocks were kept on corn meal-molasses-agar-yeast medium at 25°C. The wild-type strain employed in these studies was Canton-S. The per^S strain was a descendant of the original short period (~19 h) mutant (Konopka and Benzer, 1971). The *w disco*^{2} *f* stock was as described in Steller *et al.* (1987); the *w* allele in this strian is w^{1118} and the *f* allele is f^5. $disco^{1656}$ was also as described (Heilig *et al.*, 1991).

The per^S *disco*^{2} *f* stock was generated by crossing *w disco*^{2} *f* males to per^S females; the resulting heterozygous females were crossed to wild-type males, and w^+ *f* recombinant progeny were collected and tested for behavioral rhythms. Those progeny which were arrhythmic in DD (*disco*) were expanded into individual stocks. The presence of the per^S allele was determined by the presence of a *Mae*II restriction site: primers from 4657–4673 and 5283–5264 were used to obtain the per^S region via polymerase chain reaction (PCR) of genomic DNA; genomic DNA was prepared as described previously (Hamblen *et al.*, 1986). One fly equivalent of DNA was used for PCR according to the following PCR conditions: 1 min at 94°C, 2 min at 55°C and 3 min at 72°C for 30 cycles. All PCR reagents were from GeneAmp kit (Perkin Elmer Cetus). These PCR fragments were restricted with *Mae*II, which gave rise to 316 bp and 310 bp fragments for wild-type; and 310 bp, 185 bp and 131 bp fragments for per^S (cf. Yu *et al.*, 1987).

RNase protection assays

Flies were collected at various times during LD cycles or in DD. For each time-point, heads were isolated and RNA was extracted. RNase protections were performed as described previously (Hardin *et al.*, 1990). In all cases antisense *per* 2/3 probe was used to measure *per* RNA abundance, and antisense ribosomal protein 49 (RP49) was used as a measure of the relative amount of RNA in each sample. Quantitation was done by densitometrically scanning autoradiographs using a Microtek MSF-300GS scanner. Each protection assay was repeated at least twice with similar results.

LD behavioral monitoring

All behavioral assays were performed essentially as described previously (Hamblen *et al.*, 1986), except that a next-generation version of the activity monitoring device (Trikinetics Drosohila Activity Monitors) was used. Individual flies were first entrained for 2 days in 12 h light–12 h dark cycles, the monitored for locomotor activity for 5 days in the same light–dark cycle. Lights went on at noon and off at midnight during these experiments. The data were then analyzed using an 'education' program as described (Hamblen-Coyle *et al.*, 1989; Dushay *et al.*, 1990). Each set of eduction was done at least three separate times with similar results.

Acknowledgements

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References

Dushay,M.S., Rosbash,M. and Hall,J.C. (1989) *J. Biol. Rhythms*, **4**, 1–27.
Dushay,M.S., Konopka,R.J., Orr,D., Greenacre,M., Kyriacou,C.P., Rosbash,M. and Hall,J.C. (1990) *Genetics*, **125**, 557–578.
Ewer,J. (1990) Ph. D. thesis, Brandeis University.

Ewer,J., Hamblen-Coyle,M., Rosbash,M. and Hall,J.C. (1990) *J. Neurogenet.*, **7**, 31–73.
Hall,J.C. and Rosbash,M. (1987) *J. Biol. Rhythms*, **2**, 153–178.
Hall,J.C. and Rosbash,M. (1988) *Annu. Rev. Neurosci.*, **11**, 373–393.
Hamblen,M., Zehring,W.A., Kyriacou,C.P., Reddy,P., Yu,Q., Wheeler,D.A., Zwiebel,L.J., Konopka,R.J., Rosbash,M. and Hall,J.C. (1986) *J. Neurogenet.*, **3**, 249–291.
Hamblen-Coyle,M., Konopka,R.J., Zwiebel,L.J., Colot,H.V., Dowse,H.B., Rosbash,M. and Hall,J.C. (1989) *J. Neurogenet.*, **5**, 229–256.
Handler,A.M. and Konopka,R.J. (1979) *Nature*,**279**, 236–238.
Hardin,P.E., Hall,J.C. and Rosbash,M. (1990) *Nature*, **343**, 536–540.
Heilig,J.S., Freeman,M., Laverty,T., Lee,K.J., Campos,A.R., Rubin,G.M. and Steller,H. (1991) *EMBO J.*, **10**, 809–815.
Helfrich,C. (1986) *J. Neurogenet.*, **3**, 321–343.
Helfrich,C. and Engelmann,W. (1983) *Physiol. Ent.*, **8**, 257–272.
Helfrich,C., Cymbrowski,B. and Engelmann,W. (1985) *Chronobiol. Int.*, **2**, 19–32.
Konopka,R.J. and Benzer,S. (1972) *Proc. Natl. Acad. Sci. USA*, **68**, 2112–2116.
Konopka,R., Wells,S. and Lee,T. (1983) *Mol. Gen. Genet.*, **190** , 284–288.
Page,T.L. (1982) *Experienta*, **38**, 1007–1013.
Petersen,G., Hall,J.C. and Rosbash,M. (1988) *EMBO J.*, **7**, 3939–3947.
Rosbash,M. and Hall,J.C. (1989) *Neuron*, **3**, 387–398.
Siwicki,K.K., Eastman,C., Petersen,G., Rosbash,M. and Hall,J.C. (1988) *Neuron.*, **1**, 141–150.
Steller,H., Fischbach,K.F. and Rubin,G.M. (1987) *Cell*, **50**, 1139–1153.
Truman,J.W. (1976) *Photochem. Photobiol.*, **23**, 215–225.
Young,M.W., Bargiello,T.A., Baylies,M.K., Saez,L. and Spray,D.C. (1989) In Jacklet,J,W. (ed.), *Neuronal and Cellular Oscillators*. Marcel Dekker, New York, pp. 529–542.
Yu,Q., Jacquier,A.C., Citri,Y., Hamblen,M., Hall,J.C. and Rosbash,M. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 784–788.
Zerr,D.M., Rosbash,M., Hall,J.C. and Siwicki,K.K. (1990) *J. Neurosci.*, **10**, 2749–2762.
Zwiebel,L.J., Hardin,P.E., Liu,X., Hall,J.C. and Rosbash,M. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3882–3886.

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