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Peripheral clocks and their role in circadian timing: insights from insects

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Impressive advances have been made recently in our understanding of the molecular basis of the cell-autonomous circadian feedback loop; however, much less is known about the overall organization of the circadian systems. How many clocks tick in a multicellular animal, such as an insect, and what are their roles and the relationships between them? Most attempts to locate clock-containing tissues were based on the analysis of behavioural rhythms and identified brain-located timing centres in a variety of animals. Characterization of several essential clock genes and analysis of their expression patterns revealed that molecular components of the clock are active not only in the brain, but also in many peripheral organs of *Drosophila* and other insects as well as in vertebrates. Subsequent experiments have shown that isolated peripheral organs can maintain self-sustained and light sensitive cycling of clock genes *in vitro*. This, together with earlier demonstrations that physiological output rhythms persist in isolated organs and tissues, provide strong evidence for the existence of functionally autonomous local circadian clocks in insects and other animals. Circadian systems in complex animals may include many peripheral clocks with tissue-specific functions and a varying degree of autonomy, which seems to be correlated with their sensitivity to external entraining signals.

Keywords: circadian clock; *Drosophila melanogaster*; multi-oscillatory system

1. INTRODUCTION

Individual organisms display a multitude of behavioural, physiological and molecular rhythms, challenging us with the question about the organization of animal circadian systems. Early in the course of circadian research experimental evidence suggested that the internal temporal order is not achieved by the workings of one central clock but rather that 'the organism comprises a population of quasi-autonomous oscillatory systems' (Pittendrigh 1960, p. 165). Insects provide an excellent model system in which to explore multi-oscillatory circadian organization. They possess a rich repertoire of overt rhythmic activities such as ecdysis, foraging, courting, mating and oviposition (Brady 1974; Saunders 1982). Studies of multiple overt rhythms in individual animals demonstrated that they do not submit to one central command but must be driven by functionally separable oscillators. For example, our honoree, Dr David Saunders, has reported relative independence of different rhythms with respect to period and phase in the fleshfly, *Sarcophaga* (Saunders 1986). Vertebrates show similar phenomena: simultaneous examination of behavioural and physiological rhythms in mammals revealed cases when separately monitored variables show oscillations with independent period, a state called internal desynchronization (Moore-Ede *et al.* 1982). Such data are compatible with the existence of self-sustained oscillators maintaining independent periods in the absence of daily entrainment.

Work on insects suggested that circadian oscillators in complex animals are not confined to structures associated with the central nervous system (CNS) but may reside in non-innervated peripheral organs (Giebultowicz 1999). An early example is the self-sustained and photoreceptive clock in the moth reproductive system that controls tissue-autonomous rhythms of sperm release from testes (Giebultowicz *et al.* 1989). In cockroaches (Weber 1995) even a piece of epidermis cultured *in vitro* displays daily rhythm in cuticle secretion!

Another line of evidence suggesting the existence of multi-oscillatory circadian systems emerged from studying spatial patterns of clock-gene expressions first in *Drosophila* (Hall 1995; Giebultowicz 2000) and later in vertebrates (Whitmore *et al.* 1998; Yamazaki *et al.* 2000). Rhythmic activities of clock genes and their products are found in a surprisingly broad range of organs. Clock molecules cycle in many loci within and outside of the CNS, in tissues involved in reproduction, metabolism and excretion. This strongly suggests that, besides imposing temporal restriction on behaviour, circadian clocks may be involved in coordinating many physiological processes in a tissue-autonomous fashion (Brown & Schibler 1999; Giebultowicz 1999). However, many of the newly discovered peripheral clocks remain unassigned in their biological functions and it will be exciting to determine the nature of the output rhythms that they generate.

A question that is just beginning to be addressed by chronobiologists is how are multi-oscillatory circadian systems organized to ensure the internal temporal order of the organism? This review (or rather a 'preview', considering how little we know about the subject matter) describes current knowledge on the organization of circadian system in *Drosophila melanogaster* and other insects and provides insights into the similarities and differences

of circadian organization that have been recently uncovered in the vertebrate world. A picture that emerges from the experimental data suggests that nature has adopted variable relationships between different body clocks, depending on the physiological and environmental context, and the evolutionary position of the given organism.

2. THE MOLECULAR COMPONENTS OF CIRCADIAN CLOCKS

The molecular basis of circadian time-keeping has been the subject of several recent reviews (Dunlap 1999; Giebultowicz 2000; Reppert & Weaver 2000; Scully & Kay 2000). Nevertheless, a brief summary is given here to introduce the genes that will be discussed in this review with respect to their functions and expression patterns in the peripheral tissues. Studies in *D. melanogaster* have greatly advanced our understanding of circadian rhythms at the molecular level. To date, at least seven fruitfly genes have been described that participate in the central oscillator controlling overt circadian behaviours (for details, see Rosato & Kyriacou 2001). Two most intensely studied clock genes in *D. melanogaster* are *period* (*per*) and *timeless* (*tim*). Both genes encode RNAs that cycle with a circadian rhythm, such that RNA levels are high at the end of the day/beginning of the night (Hardin *et al.* 1990; Sehgal *et al.* 1995). The *per* and *tim* gene products, proteins PER and TIM, also cycle and begin accumulating in the middle of the night. PER and TIM bind one another to form heterodimers, which are transported into the nucleus. Each protein is required for nuclear transport of the other; i.e. in *per* and *tim* null mutants, TIM and PER, respectively, are restricted to the cytoplasm (Hunter-Ensor *et al.* 1996; Saez & Young 1996). In wild-type flies, the abundance of both proteins peaks in the cell nuclei late at night. Because both PER and TIM lack conventional DNA binding domains and have never been shown to associate directly with DNA, models have postulated that these proteins associate with transcriptional activators and sequesters them. Transcriptional activators of *per* and *tim* have been recently described; they are encoded by the *dClock* (*dClk*) and *cycle* (*cyc*) genes, and flies mutant at either locus express very low levels of *per* and *tim* RNA (Allada *et al.* 1998; Rutila *et al.* 1998). Furthermore, cell culture studies show that PER and TIM act by inhibiting the activity of CLK/CYC (Darlington *et al.* 1998) which brings *tim* and *per* mRNA to low levels. At the same time, PER and TIM are necessary to stimulate transcription of *dclk* (Glossop *et al.* 1999). This complicated molecular loop perpetuates itself in the absence of external inputs with a *ca.* 24 h period. However, external inputs, mainly in the form of day/night cycles, bring this loop (as well as clock-controlled output rhythms) into synchrony with the outside world. This is accomplished by little-understood circadian photoreceptors such as cryptochrome (for a recent review, see Hall 2000; see also Foster & Helfrich-Förster 2001).

Fruitfly clock genes have functional homologues in vertebrates (Reppert & Weaver 2000), and basic principles of their interactions via feedback loops are conserved from insects to mammals (Glossop *et al.* 1999; Shearman *et al.* 2000). Another conserved feature of circadian mechanisms is that they are cell-autonomous; single cells dissociated *in vitro* can keep track of time even in complex multicellular animals (Michel *et al.* 1993; Welsh *et al.* 1995), although *in vivo* such cells are usually grouped into so-called timing centres.

3. MULTIPLE TISSUES EXPRESS CLOCK GENES

The question about the distribution of the timing centres in the body was addressed in the past by relating rhythmic output to specific organs or cells. The drawback of this approach is that one can only study those clocks for which overt measurable output rhythms are identified. The knowledge of the circadian feedback loop gives us a more objective tool to search for putative clocks, by identifying cells and organs that display rhythmic expression of clock genes. The 'veteran' of the insect clock genes, *period*, originally identified in *D. melanogaster* (Konopka & Benzer 1971), has been most thoroughly characterized in terms of spatial expression patterns in fruitfly tissues (for a review, see Hall 1995). Cycling of *per* mRNA and PER protein was detected in specific areas of the CNS, including visual photoreceptors, several subsets of brain neurons and groups of glial cells (Siwicki *et al.* 1988; Zerr *et al.* 1990; Kaneko *et al.* 1997), suggesting that there may be many oscillators within the CNS. Rhythmic activities of *per* gene were found also in many peripheral tissues, including gut, excretory system and testes (Liu *et al.* 1988; Saez & Young 1988; Hege *et al.* 1997; Plautz *et al.* 1997). Because PER must associate with TIM to form a functional component of the feedback loop, it is important to demonstrate co-localization of both proteins in putative oscillator sites. In most CNS loci where PER protein is detected, TIM protein displays coordinated nuclear cycling (Hunter-Ensor *et al.*, 1996; Kaneko & Hall 2000). The same is also true for renal (Malpighian) tubules (Giebultowicz & Hege 1997) and many other peripheral organs, such as the alimentary tract, rectum, fat body and parts of the reproductive system (Giebultowicz *et al.* 2001). However, some tissues *of D. melanogaster*, including epidermis, skeletal muscles and tracheal epithelium, do not show detectable levels of either PER or TIM (Giebultowicz *et al.* 2001) demonstrating that *per*/*tim*-based oscillators are not found 'throughout *Drosophila*' (Plautz *et al.* 1997), but rather are limited to specific, albeit numerous, organs.

Cloning of the *per* homologue in other insects (Reppert *et al.* 1994) has opened the way to analyse the activity of this gene outside of *D. melanogaster* and revealed *per* expression in both CNS and in peripheral tissues of moths. There are circadian oscillations of *per* mRNA and protein in the larval gut of the silkworm, *Antheraea pernyi* (Sauman & Reppert 1998), and in the testes–vas deferens complex of the codling moth, *Cydia pomonella* (Gvakharia *et al.* 2000). Those epithelial tissues show periodic nuclear localization of PER in agreement with the fruitfly model for the clock, but in contrast to neurons in the silkworm central brain where PER remains in the cytoplasm at all times (Sauman & Reppert 1996).

Peripheral expression of clock genes is not limited to insects; components of the circadian feedback loop, such as *clk* and *per* genes, are expressed in many mammalian internal organs (King *et al.* 1997; Tei *et al.* 1997). These

findings were surprising to many chronobiologists, since most of the known mammalian rhythms disappear when the central clock in the suprachiasmatic nucleus (SCN) is removed (Underwood *et al.* 1997). The expression of specific clock genes in vertebrate peripheral organs is rhythmic and, therefore, probably related to timing. From fish to mammals, mRNAs derived from *Bmal1* (the vertebrate equivalent of *cyc*) and *per* genes show cycling in organs such as heart, lungs, kidney and testis (Oishi *et al.* 1998; Whitmore *et al.* 1998; Zylka *et al.* 1998). Thus, the molecular components of the circadian clock cycle in peripheral organs of phylogenetically distant complex animals, attesting further to the conservancy of biotiming principles that was already demonstrated in formal features of circadian rhythms and in homologies of clock genes.

4. SELF-SUSTAINED AND PHOTORESPONSIVE OSCILLATORS IN PERIPHERAL TISSUES

Peripheral tissues, which rhythmically express clock genes, may be involved in circadian timing; but to be promoted to a *bona fide* clock status, they need to go through several qualifying steps. An important criterion qualifying tissues as having independent pacemaking function is the ability to maintain self-sustained circadian oscillations when explanted *in vitro*. A second criterion that would make self-sustained clocks potentially independent from the rest of the body is the ability to be entrained directly by environmental signals. One of the first tissues identified in an insect that fulfilled both criteria is the testes–vas deferens complex in the gypsy moth in which output rhythms of sperm release (discussed below) continue and are light-entrainable *in vitro* (Giebultowicz *et al.* 1989). Recently, an avalanche of putative oscillators have been demonstrated in *D. melanogaster* transformed with luciferase, which acts as a real-time reporter for *per* and *tim* activities (Brandes *et al.* 1996; Stanewsky *et al.* 1998). Owing to the fact that *per*-expressing organs of transformed flies produce measurable light *in vitro*, several self-sustained and light-entrainable oscillators were identified in peripheral tissues. One such tissue is the ring gland, which produces the insects' moulting hormone ecdysone. In fly pupae, *per* gene and protein are rhythmically expressed in the ring gland and this expression continues *in vitro* (Emery *et al.* 1997). *per*-driven rhythmic luciferase activity was detected also in chemosensory structures located on flies' antennae, proboscis, wing margin and legs (Plautz *et al.* 1997). Both studies demonstrated that *per* oscillations can be light-entrained *in vitro*. The list of self-sustained and photoreceptive clocks in *D. melanogaster* includes renal tubules and rectum. These segments of the fly excretory system display robust oscillations of both *per*- and *tim*-driven luciferase activities *in vitro* (Giebultowicz *et al.* 2000).

Insects are certainly not the only animals in which self-sustained and photoreceptive oscillators have been found outside of the central brain. It is well documented that pineal glands and retinas of various vertebrates rhythmically produce melatonin, and that those rhythms persist and phase shift *in vitro* (Underwood *et al.* 1997). The range of vertebrate tissues harbouring putative oscillators has been recently extended to many peripheral organs. Persistent cycling of the *clock* gene was demonstrated in explanted livers, kidneys and hearts of zebrafish (Whitmore *et al.* 1998) and these oscillations shifted in response to changes in environmental light cycles applied *in vitro* (Whitmore *et al.* 2000). A crowning touch to the developing story of peripheral oscillators comes from mammals. Cycling of clock genes and clock-controlled genes occurs in mammalian cultured fibroblasts (Balsalobre *et al.* 1998). A recent report demonstrated rhythms of *per*-luciferase in livers, skeletal muscles and lungs explanted from transgenic rats (Yamazaki *et al.* 2000). What seems to set apart peripheral oscillators in mammals from those in insects and lower vertebrates is that the latter are photoresponsive while the former are not. For the comparative discussion of light-entrainment in insect and mammalian circadian systems see review by Foster & Helfrich-Förster 2001.

5. WHERE IS THE PHYSIOLOGY?

Data from *in vitro* experiments suggest that many peripheral organs of insects and vertebrates have the potential to function as independent oscillators. Because they were identified on the basis of cycling expression of clock genes, most of them are 'orphan oscillators' without known output rhythms. Promotion of these oscillators to a clock status should depend on the identification of the rhythmic outputs and the understanding of their relevance to the organism's physiology.

There are few cases where the physiological role of peripheral clocks is relatively well understood. One prominent example was found in moths in which an autonomous circadian system in male reproductive organs orchestrates orderly succession of physiological processes vital for the survival of the species (Giebultowicz *et al.* 1989). The testis–vas deferens complex of male moths displays many coordinated rhythms associated with a daily cycle of sperm release and maturation, as schematically depicted in figure 1. Clones of differentiated spermatozoa (sperm bundles) are released from the testis into the vas deferens by penetrating the epithelial barrier separating the two organs during the circadian gate at the end of the day. Exit channels are formed in the epithelial barrier and the sperm bundles are released from the testes, leaving behind the cyst cells within which they developed (Giebultowicz *et al.* 1997). The peak of sperm accumulation in the vas deferens lumen is correlated with the release of glycoproteins from the apical portion of the vas deferens epithelium (Riemann & Giebultowicz 1991). Ultrastructural studies suggest that the secretory materials interact with the sperm and are involved in sperm maturation (Riemann & Thorson 1971; Riemann & Giebultowicz 1992). After night-time retention in the vas deferens lumen, sperm is transferred to the seminal vesicles due to the morning increase in the intensity of contraction of the vas deferens wall (Giebultowicz *et al.* 1996). The importance of circadian coordination of sperm release and maturation is manifested dramatically in constant light, which disrupts all rhythms and leads to male sterility (Giebultowicz *et al.* 1990).

All the rhythms described above may be driven by the *per*-based circadian mechanism; *per* mRNA and PER protein are rhythmically expressed in the secretory and

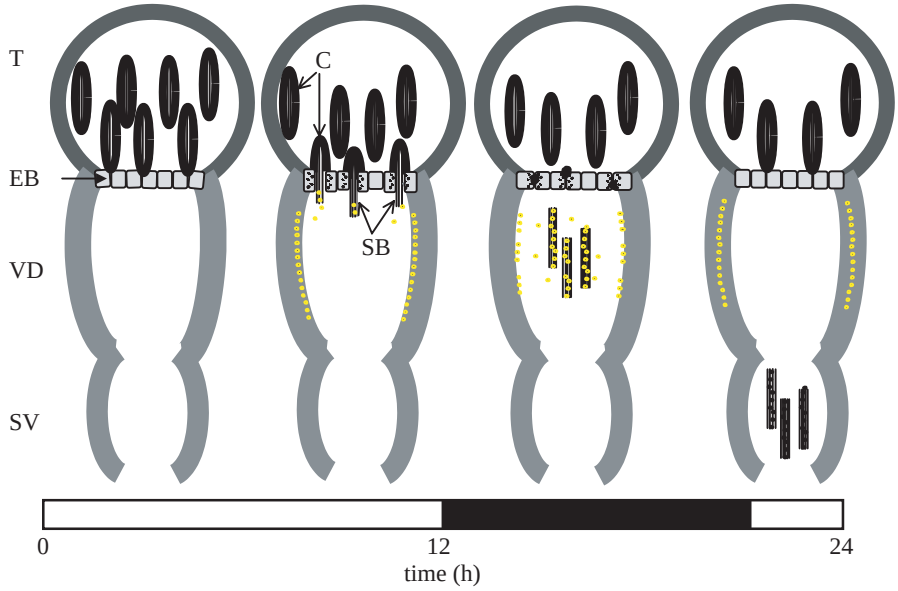

Figure 1. Scheme illustrating the daily cycle of sperm release and associated cellular rhythms in moths. A portion of mature sperm bundles accumulated in the testis (T) is released into the vas deferens (VD) once a day within few hours before lights-off. Sperm bundles (SB) leave the testis through the exit channels formed between the epithelial barrier cells (EB), which separate the testis from the vas deferens lumen. During the exit, cyst cells (C) surrounding sperm bundles degenerate and their remnants are phagocytosed by the barrier cells. Sperm bundles that are released into the vas deferens lumen are coated with material secreted from the vas deferens epithelium. Subsequently, sperm bundles are transferred into the seminal vesicles (SV) within a few hours after lights-on due to increase in myogenic contractions of the VD wall. Daily batches of released sperm accumulate in the duplex, a storage organ from which bundles are retrieved during mating. Horizontal bar, day (white) and night (black) portions of the daily cycle.

muscle cells of the vas deferens and in cells forming the epithelial barrier between the testes and the vas deferens (Gvakharia *et al.* 2000). To our knowledge, it is not yet understood to what degree clock-containing cells communicate with each other in this diffused peripheral circadian system. Although *per* is broadly expressed in the vas deferens, it is not ubiquitous in the reproductive system; no activity of this clock gene was detected in the developing germ cells or the testis wall surrounding them. Interestingly, a similar pattern of clock-gene expression was observed in *Drosophila* reproductive tract, and fruitfly mutants lacking the *period* gene show lower reproductive potential (Beaver *et al.* 2001). Thus, circadian control of sperm release and maturation may operate in insects other than moths and, perhaps, beyond insects, given the expression of clock genes in mammalian gonads (Zylka *et al.* 1998).

There are other cases where physiology and clock genes begin to merge in *Drosophila*. For example, a defined output function has been assigned to the peripheral oscillators in the chemosensory hairs on the fly antennae. These organs display a rhythm in electrophysiological responses to two different classes of olfactory stimuli. Olfactory rhythms are driven by clock genes expressed locally in the antenna, although it is not yet known whether neuronal or epithelial components of sensory hairs are involved in timekeeping (Krishnan *et al.* 1999). Olfactory rhythms may be common in insects: tsetse flies show daily modulation in the perception of host odours (Van der Goes van Naters *et al.* 1998), and many moths have daily rhythms in pheromone sensitivity (Raina & Menn 1987).

The links between clocks and physiological outputs include several intermediate steps leading from clock genes via other transcription factors to the effector genes that are responsible for cellular physiology. Some steps are known in both central and peripheral oscillators: a handful of rhythmic transcription factors and effector genes has been identified (for a review, see Brown & Schibler 1999; Jackson *et al.* 2001), but in no case do we understand the whole clock-to-physiology cascade. Given this paucity of information it is not clear whether circadian clocks control diverse cellular processes in different tissues or whether there are specific rhythmic aspects of cellular physiology that are shared by many cell types. Convergent clock-controlled output pathways seem to occur in neurons and epithelial cells. For example, the mammalian transcription factor DBP shows a circadian rhythm in both central (SCN) and peripheral (liver) oscillators; generation of this rhythm at the transcription level seems to involve the CLOCK protein (Ripperger *et al.* 2000). In *D. melanogaster*, clock-controlled oscillatory expression of the gene *takeout*, which is implicated in the control of feeding, occurs not only in the brain (So *et al.* 2000) but also in segments of the alimentary tract (Sarov-Blat *et al.* 2000).

6. CIRCADIAN ORGANIZATION: AUTONOMY OR HIERARCHY?

The molecular and physiological evidence for multi-oscillatory circadian systems in complex animals poses the following question: how are such systems organized to ensure synchronization of different body functions? The

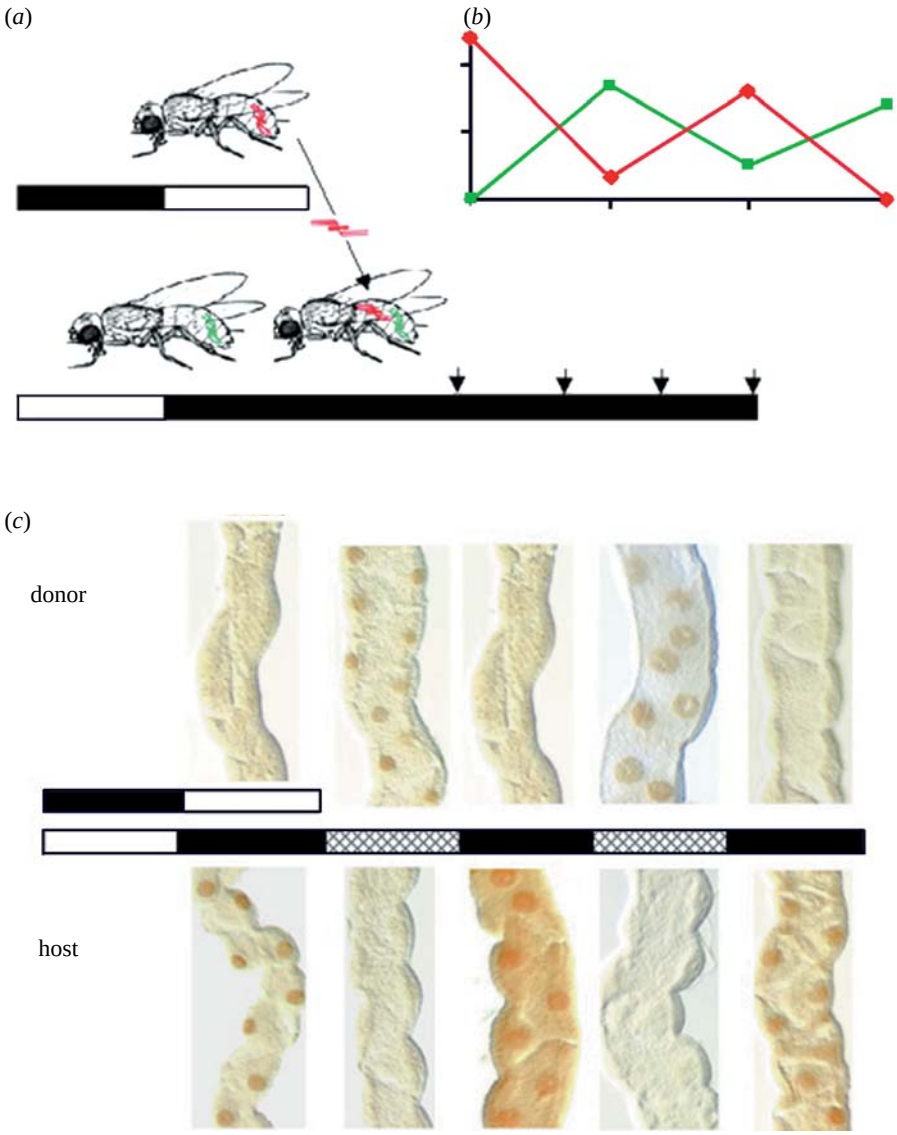

Figure 2. Expression of TIM protein in the transplanted Malpighian tubules (MTs) of *Drosophila melanogaster*. (*a*) Scheme explaining the experiment in which tubules were transplanted from flies reared in light–dark (LD) cycles to flies reared in reverse LD. (*b*) Time course of TIM nuclear staining in donor (red) and host (green) tubules following transplantation. (*c*) Representative examples of TIM signal in MTs before the operation and in host and donor tubules at 12 h intervals after transplantation (arrows in (*a*)). White and black bars, time when lights were on and off, respectively; shaded bars, subjective day when lights would be on in LD but were off in this experiment. Each bar represents 12 h. After Giebultowicz *et al*. (2000).

fact that peripheral clocks are competent to oscillate on their own *in vitro* tells us little about their relationships with each other and with the central clock *in vivo*. Conceptually, it is possible that peripheral clocks may operate independently within the body; the synchrony of the circadian system would then result from their ability to be directly entrained by external environmental cycles. On the other hand, peripheral clocks could be coupled to each other and/or sensitive to internal synchronizing signals generated by the central clock. The data from studies that addressed these possible models suggest that both may be valid, even in one organism.

(a) *Independent clocks in insects and beyond*

In *D. melanogaster*, a group of lateral neurons which control the output rhythm of locomotor activity (Helfrich-Förster *et al*. 1998) is considered as the central clock due to its behaviour-regulating role. However, there is no evidence to suggest that the clock in the lateral neurons dominates other clocks either in the head or periphery. Clock molecules cycle with similar phases in brain and peripheral tissues. For example, rhythms of *per*, *tim* and their respective proteins do not show significant phase lag in Malpighian tubules and rectums relative to the brain clocks, when examined *in vivo*, in decapitated flies or *in vitro* (Giebultowicz & Hege 1997; Hege *et al*. 1997; Giebultowicz *et al*. 2000). Even more surprisingly, some of the peripheral clocks appear to phase-lead the head clocks by a few hours with respect to the cycling of clock genes. We measured the activities of *per*-luciferase and *tim*-luciferase reporters in cultured *Drosophila* testes and observed that both increased a few hours earlier in isolated testes compared with isolated heads (J. M. Giebultowicz and R. Stanewsky, unpublished data). This is consistent with the observation that the levels of PER and TIM proteins increase earlier during the night in

testes compared with the brain (B. O. Gvakharia, personal communication).

The second argument against hierarchical organization of the fly circadian system comes from monitoring the resetting of peripheral clocks in the Malpighian tubules. The phase shift in the oscillation of the *per* gene in Malpighian tubules, following the reversal of light–dark (LD) cycles, occurs with a very similar time-course in intact and decapitated flies (Hege *et al.* 1997). These data suggest that the central clock does not mediate the resetting of the peripheral clock, but rather, Malpighian tubule clocks seem to be directly entrained by environmental cycles. One could argue, however, that in the absence of environmental cues humoral factors secreted by the central clock might regulate the phases of peripheral oscillations. To test for humoral factors, we monitored cycling of the TIM protein in Malpighian tubules transplanted into host flies entrained to an opposite LD cycle and kept in constant darkness after the surgery (figure 2). Under those conditions, TIM protein in the donor tubules cycled out of phase relative to host tubules, despite the fact that both sets of tubules were sharing the same hormonal milieu (Giebultowicz *et al.* 2000). This, to our knowledge, is the strongest evidence to date supporting the idea that specific peripheral clocks in the fly may operate as totally autonomous units.

Another piece of evidence suggesting that fruitfly peripheral clocks 'ignore' hormonal milieu comes from examining the developmental regulation of clock-gene expression during metamorphosis. We determined that the activity of *per* and *tim* in various peripheral organs began at different stages of metamorphic development. For example, both *per* and *tim* became active in the rectum two days before adult eclosion, while in the Malpighian tubules they are first expressed on the day of eclosion. These data suggest that the onset of clock-gene expression in the periphery is tissue-autonomous rather than triggered by development-dependent hormonal signals in the hemolymph (Giebultowicz *et al.* 2001).

The results discussed above suggest that many peripheral pacemakers in flies have a high degree of autonomy. The same situation seems to prevail in lower vertebrates as well. In the zebrafish, the expression of the *clk* gene is rhythmic in kidney, spleen and heart, and the oscillations have similar phases *in vivo* and *in vitro* (Whitmore *et al.* 1998). Subsequent studies have shown that *clk* rhythms in zebrafish peripheral tissues are entrainable by light *in vitro* (Whitmore *et al.* 2000). The complete resetting of the *clk* mRNA rhythms *in vitro* is accomplished by the second cycle after reversal of the LD cycle, suggesting that this process is not mediated by the brain, similarly to *Drosophila* (Hege *et al.* 1997). It is not known whether self-sustained and photoreceptive clocks exist in the peripheral tissues of other vertebrates. Amphibians, reptiles and birds have such clocks in their pineal glands and retinas, but specialized photoreceptor structures may be involved in their entrainment (Underwood *et al.* 1997).

(b) *Not all clocks are independent*

There is a handful of cases suggesting that some oscillators in insects are hierarchically organized. An interesting example of clocks interacting via the humoral pathway is the brain–prothoracic glands axis in the hemipteran bug *Rhodnius prolixus*. These insects display pronounced circadian fluctuations in the levels of circulating ecdysone. Prothoracic glands of *R. prolixus* harbour photosensitive circadian oscillators that drive rhythmic release of ecdysone *in vitro*. The phase of the rhythmic output of this oscillator is different *in vivo* and *in vitro* (Vafopoulou & Steel 1998). It was shown that the clock in the brain, which rhythmically releases the hormone stimulating ecdysone synthesis (prothoracicotropic hormone, PTTH), exerts modulating effects on the clock in the prothoracic glands (Pelc & Steel 1997). Consistent with these results, there is a daily rhythm in the responsiveness of the prothoracic glands to PTTH (Vafopoulou & Steel 1999). There may be other oscillators in insects whose function is regulated from the head. Rhythmic expression of PER protein was observed in the gut epithelium of the intact silkworm larvae, but this rhythm was disrupted in decapitated larvae (Sauman & Reppert 1998).

To find compelling evidence for hierarchical organization of the circadian system one needs to make a phylogenetic leap to mammals. The suprachiasmatic nucleus (SCN) of the brain is the mammalian master oscillator controlling most behavioural and physiological rhythms. However, like other animals, mammals show rhythmic expression of clock genes in their peripheral tissues. Recent study using transgenic rats expressing luciferase under the *per* promoter examined patterns of *per*-driven light emission in explanted SCNs, livers, lungs and skeletal muscles (Yamazaki *et al.* 2000). All cultured organs expressed circadian rhythms, but cycling of *per*-luciferase in the SCN was more robust and persisted for many more days than the cycling in peripheral tissues. Two lines of evidence suggest that peripheral oscillators in rats are differently regulated than the SCN clock. First, rhythms in peripheral tissues phase-lag the SCN rhythm by 7–11 h both *in vivo* and *in vitro*. Second, the rhythm in the SCN shifts more rapidly than do the rhythms in peripheral tissues in response to advances and delays of environmental light cycles administered before organ isolation (Yamazaki *et al.* 2000). Mammalian peripheral oscillators are likely to be regulated by rhythmic humoral signals; a recent report demonstrated that glucocorticoids can shift (albeit transiently) the phase of circadian gene expression in liver, kidney and heart (Balsalobre *et al.* 2000).

(c) *Evolutionary perspective*

The limited survey presented here suggests that circadian synchronization of life functions in complex animals may involve an array of relationships between different clocks, including total and partial autonomy. The status of the peripheral clocks relative to the brain clock seems to be quite different between mammals, on the one hand, and lower vertebrates and invertebrates, on the other. In mammals, peripheral clocks appear to have lost the ability to respond to light, the most precise external resetting signal (Yamazaki *et al.* 2000). Inevitably, these peripheral oscillators may be expected to submit to phase resetting signals derived from a photosensitive central timing system. However, in insects and lower vertebrates, which retained light-entrainment pathways in their clock-harbouring tissues, circadian coordination of physiological systems may be achieved through the direct

entrainment of light sensitive clocks by environmental signals. Support for this notion can be gleaned from an evolutionary perspective. Clocks are found in bacteria and unicellular eukaryotes (Edmunds 1988; Dunlap 1999); as simple organisms evolved into tissues of higher animals, these descendent tissues could have easily retained their rhythmic capacity. The link between the environment and insect peripheral clocks is very strong. Arguably, the role of a central pacemaker is to reschedule the rhythms of its subordinate organs so they can remain in synchrony with environmental changes. Yet, if these organs can respond effectively to these changes, a central command might be superfluous. Thus there would be little selection pressure for an autocratic pacemaker to take over control of a tissue that has retained its phylogenetically ancient circadian function, especially if this tissue's response to environmental cues is sufficient for adaptation.

Although intuitively not obvious, it is conceivable that a collection of independently entrained clocks could fine-tune the physiological state of an insect. For example, at the time when the clock in the brain stimulates locomotor activity via its neuronal and hormonal outputs, an intestinal clock may stimulate the production of digestive enzymes in anticipation of foraging, and a clock in the renal system may stimulate excretory machinery in anticipation of the increased load of metabolic waste.

There seems to be no simple answer to the question about interactions between multiple oscillators in complex animals. As we progress in probing the circadian organization in different creatures, we are likely to discover a whole spectrum of relationships between clocks in different organs depending on their physiological functions and the phylogenetic position of species in which they operate.

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